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The mortality and morbidity of prostate cancer (CaP), the commonest cancer in men in Western society, are largely caused by the spread of CaP to bone. How this occurs, and the interactions between CaP cells and bone are poorly understood. Specific mutations of the tumor suppressor gene, *p53*, that occur in CaP may cause disease progression. To test this, we have generated cell lines derived from the human LNCaP cell line that stably express wild type or mutant *p53*.

Purpose: Using generated lines, to test whether *p53* mutations affect establishment and growth of experimentally-induced CaP in the bone.

Scope: Our initial studies in tissue culture will show whether factors produced by the variant LNCaP lines alter the normal processes of bone remodeling and angiogenesis.

Results/Progress: Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells with different p53 mutations showing differentiation stage dependent effects. We have perfected the technique for implanting CaP cells in the bone of immunologically deficient mice, but further *in vivo* experiments will be performed.

Significance: Further studies will provide information about how specific mutations of *p53* found in patients with clinical disease impact on progression, and could lead to the development of new therapeutic strategies.

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THE ROLE OF p53 MUTATIONS IN METASTASIS OF PROSTATE CANCER TO BONE

Annual Report, DAMD17-02-1-0108, January 2004

INTRODUCTION:

The mortality and morbidity of prostate cancer (CaP), the most common cancer in men in Western society, are largely due to bony metastases, yet how this occurs, and the cellular interactions between CaP cells and the bone microenvironment, are poorly understood. The **subject** of this work is to study the role of *p53* mutations in the metastasis of prostate cancer to bone. We have generated a series of cell lines derived from the prostate cancer cell line, LNCaP, that stably expresses wild type *p53* (Wt) or *p53* mutants (F134L, M237L and "hotspot" R273H), detected in different clinical prostate cancers. For comparison, we also have the Empty line, stably transfected with the plasmid vector, pRcCMV2 and the untransfected parent line.

Based on the hypothesis that specific *p53* mutations found in prostate cancers are involved in promoting tumor progression of CaP, our **purpose** is to use these lines to test the role of *p53* mutations in the establishment and growth of experimentally-induced CaP bone metastases. The F134L, M237L and R273H mutations are in the DNA binding domain of p53 (amino acids 102-292) and we have shown that they exhibit a dominant-negative phenotype, as determined by promoter trans-activation experiments in CaLu-6 lung carcinoma cells. However, in LNCaP prostate cancer cells, where F134L and R273H mutants were dominant-negative, the M237L mutant had a wild-type phenotype and stimulated promoter trans-activation. These data led us to hypothesise that these LNCaP p53 variants might behave differently in interactions with bone cells.

The **scope** of research involves initial studies performed *in vitro* to determine the effects of factors produced by the LNCaP parent and transfectant lines on the normal process of bone remodelling and on angiogenesis. Stable LNCaP transfectants expressing Wt or mutant *p53* will then be implanted in the tibia to assess their potential to form osteoblastic lesions, and in the heart of SCID or NOD.SCID mice, to determine their ability to spontaneously metastasize to bone. This work will provide information about how specific mutations of *p53* found in patients with clinical disease impact on disease progression, and could identify targets for further study.

BODY:

For each of the studies described below, 6 LNCaP cell lines were used. These were: LNCaP-parental, LNCaP-Empty, LNCaP-Wtp53 (overexpresses Wt p53), LNCaP-F134L (expresses mutant p53), LNCaP-M237L (expresses mutant p53) and LNCaP-R273H (expresses "hot-spot" mutant p53); for convenience, these have been abbreviated as LNCaP-P, Empty, wtp53, F134L, M237L and R273H, respectively. Elizabeth Kingsley* has been involved in producing conditioned medium, examining its effects on osteoblast cells by RT-PCR and protein analysis; Barbara Szymanska*, PhD, joined us in late June 2002, and has been involved in work on mineralization of collagen, and studies of osteoclast differentiation, and intracardiac inoculations; Lara Perryman*, was appointed on the 1st of May 2002, and has been involved in cell culture, particularly of endothelial cells, intratibial injection of mice and analysis of procedures. Julie Blair (nee Brown)* initiated the osteoblast studies and the bone injections and has helped with each of these tasks. (* These staff have salaries from the grant; Dr Blair has only 20% of her salary from this grant). By the end of 2002, we had accomplished all of Task 1A, but decided to expand some the data sets obtained (see below); Tasks 1B, 1C and 2A were also initiated and problems that were encountered were

resolved; Tasks 1D, 2B, 3A and 3B were started, as anticipated. In addition, we initiated experiments for Task 1E, programmed for months 13-18.

At this stage of the project (end of 2003), we have completed and extended Task 1A. For Tasks 1B and 1C, we have completed all sample production and sample processing, partially completed the RT-PCR analysis, and identified and obtained the kits required for protein analysis or, where necessary, developed new methodology (see below). We have also performed additional experiments for task 1C, where we have begun to analyse the gene expression profile of the cell lines using cDNA arrays. Tasks 1D and 1E have been completed. We have also completed and expanded Task 2A, and initiated 2B, but Task 3 has been held up because of illness that occurred in the SCID mice that were the hosts for intratibial injections of the cell lines of interest. Task 4 has been initiated. The following report details the results obtained so far.

TASK 1A: Determine the effects of transfected LNCaP cells on osteoblast proliferation *in vitro*

Objective:

The MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation, respectively. These were treated with conditioned medium (CM) produced by the six p53-variant LNCaP prostate cancer cell lines (described above), in order to determine any effects upon cell proliferation.

Methods:

i. Production of CM: CM was produced from the six LNCaP lines, LNCaP-P, Empty, wtp53, F134L, M237L, R237H. Cells were grown to ~70% confluency in 150 cm^2 flasks, then washed twice with phosphate buffered saline, pH 7.2 (PBS), and fed 25 mL of serum-free (SF) T-medium (see Appendix 1). After 24 hours, the CM were collected, centrifuged to remove any cellular debris, aliquotted, and stored at -20°C. This method was used for the production of large quantities of CM (see below). For experimental method *xv* the CMs were pooled as shown in Appendix 4.

ii. New methodology for production of CM: Task 1A required the examination of the effects of CM upon the osteosarcoma cell lines in a series of dose response experiments. It was further decided to compare directly the effects on the osteosarcoma cells of similar doses of the various CM. In order to do this, the methodology by which the CMs were produced was refined. Untransfected LNCaP-P and the transfected LNCaP cells were grown to 70% confluency under standard tissue culture conditions and were then passaged using trypsin. The cells were replated at 8 x 10^6 cells/ 150 cm^2 flask and allowed to adhere for two days. Then, the media were removed and the cell layers gently washed twice with pre-warmed PBS. Serum-free T-medium was dispensed into each flask and the cells returned to the incubator for 24 hours. The conditioned medium (CM) for each cell line was collected, pooled and centrifuged to remove cell debris. The CMs were then stored as one-use aliquots at –20 °C until required.

iii. Determination of optimal seeding for osteosarcoma cell lines: The appropriate seeding numbers for the MG-63, U-2 OS and Saos-2 cell lines were determined using cell density experiments. Cells were seeded into 96-well plates, in 100 µL/well, at 100-10000 cells/well, and fed with 100 µL of fresh medium on the next two days. On the third day, the cells were microscopially examined to determine a seeding number for each line which would give a confluency of ~ 70% by day 3. This would allow any proliferative effects of the CM to be detected. For the U-2 OS line, 1000 cells/well

were chosen; for the MG-63 and Saos-2 lines, 2000 cells/well. When the assays were extended to an 8 day protocol (see *Results*, below), this task was repeated, and seeding numbers of 250, 300 and 750 cells/well chosen for the U-2 OS, MG-63 and Saos-2 lines, respectively.

iv. Effects of CM on proliferation of osteosarcoma cell lines: Proliferation assays were carried out in quadruplicate for each of the three osteosarcoma lines, in the presence of all six CM at various concentrations. As LNCaP cells grow in T-medium, and the osteosarcoma lines in DMEM, the CM solutions were adjusted so that all cells were exposed to a similar concentration of the T-medium supplements. This was done by making up the appropriate volume of CM in T medium to 50% of the final volume, and diluting with an equal volume of DMEM containing 20% FBS, giving a final concentration of 10% FBS. Cells were seeded into 96-well plates at the appropriate density on Day 0. Initially, cells were fed with freshly prepared media solutions containing CM at various concentrations on Days 1 and 2. On Day 3, the media were replaced with phenol red-free, serum-free DMEM, and cell proliferation assessed using the WST-1 Cell Proliferation Assay (Roche; Cat. No. 1 644 807), as per the manufacturer's instructions. Optical density readings were taken at 450 nm, and the data analysed using one-way ANOVA at a confidence interval of 95%. For the 8-day protocol, the cells were fed on Days 1, 4 and 6, then assessed as described on Day 8.

Direct comparison of the effects of the different CM at the same concentration (i.e. all 6 CM at 5%, at 10%, at 25% and at 50%) was undertaken using CM produced under the revised, standardised conditions (see '*ii*, ***New methodology for the production of CM***', above), and carried out using the 8-day protocol.

Results:

No statistically significant differences in proliferation of the three osteosarcoma cell lines was seen after treatment with any CM for up to 3 days, but there were trends, most noticeably an increase in both U-2 OS and MG-63 proliferation after treatment with 10% F134L CM and a decrease in MG-63 proliferation following treatment with 50% R273H CM. These experiments were therefore repeated and the time of exposure of the osteoblast cell lines to the CM was extended to 7 days. Whilst this part of the work was outside of the scope of the nominated Task 1A, we believed that additional work could provide productive data.

Over 7 days, CM from LNCaP-P cells inhibited the proliferation of immature osteoblasts and stimulated that of mature osteoblasts. LNCaP-empty CM had little effect on osteoblastic cells. LNCaP-wtp53 CM stimulated U-2 OS cell proliferation only. LNCaP-F134L CM was anti-mitogenic for MG-63 and Saos-2 cells at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS and Saos-2 cell proliferation at intermediate doses. Treatment with LNCaP-R273H CM increased MG-63 and U-2 OS cell numbers at 5-25% when compared with 0% treatment. *, $p<0.05$ and **, $p<0.01$ when compared with 0% CM (Appendix 2, Figure 1).

When the CM were compared directly at the various concentrations, the most significant result for all three cell lines was a marked inhibition of proliferation following treatment with the R273H CM at 50%.

Discussion

The MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation. The effects on osteoblast-like cell proliferation for all three osteoblast-like cell lines were typically biphasic for most CMs. M237L CM stimulated the proliferation of U-2 OS and Saos-2 cells, whereas over lower doses, R273H was mitogenic for MG-63 and U-2 OS

cells, suggesting differentiation stage-dependent effects of these CMs. At high concentrations, however, R273H CM inhibited the proliferation of all three cells lines. F134L and empty control CMs had little effect.

The significance of these results remains to be determined. It will be necessary to determine whether the suppression of osteosarcoma proliferation following treatment with 50% R273H is due to toxicity. This is unlikely given that it does not occur across all 6 CM, suggesting that it is a true anti-proliferative effect. These data may indicate that factors secreted by prostate cells carrying the R273H 'hot spot' *p53* mutation can influence osteoblastic differentiation.

TASK 1B: Determine the effects of transfected LNCaP cells on osteoblast differentiation *in vitro*

TASK 1C: Effects of LNCaP transfectants on collagen production

(**NB:** As the early stages of the work for these two tasks were identical, all experiments were conducted simultaneously).

Objectives:

To isolate RNA and protein from homogenates of osteosarcoma cell lines treated with CM from LNCaP parent and transfected lines, in order to determine the effects of CM on markers of osteoblast differentiation. Alkaline phosphatase and osteocalcin are early- and late-stage markers of osteoblast differentiation, respectively. Type I collagen is produced by osteoblasts during the formation of new bone, while osteoprotegerin is a bone resorption inhibitor that blocks the formation and activation of osteoclasts.

Task 1B-a/Task 1C-a, c: Osteoblast differentiation assays

These experiments were performed in triplicate for all three osteosarcoma cell lines exposed to CM from the six LNCaP lines. Fifty-four experiments were carried out: each cell line was treated with the 6 CM in triplicate, with five concentrations of CM (0, 5, 10, 25 and 50%) at each of five timepoints (3, 6, 12, 24 and 48 h), giving a total of 1350 samples.

Methods:

i Production of CM: (see i above) In order to save both time and effort, large-scale production of CM from all six cell lines was undertaken in parallel with Task 1A. In total, >1000 mL of CM from each the six LNCaP cell lines were produced, aliquotted, and stored at -80°C.

v. Effects of CM on differentiation of osteoblasts: Assays were carried out as follows:

Day 0: The U-2 OS and MG-63 osteosarcoma cell lines require 3.0×10^7 cells per experiment; the Saos-2 cell line, 3.75×10^7 cells per experiment. U-2 OS or MG-63 cells were seeded into 25 x 75 cm^2 flasks in 10 mL medium, at 1.2×10^6 cells/flask (i.e. ~ 1.6×10^4 cells/cm^2), a density provided from previous studies by Julie Blair (nee Brown) that promotes differentiation rather than proliferation. Saos-2 cells posed difficulties, with unavoidable cell loss when seeded and failure to obtain complete cell removal by trypsinization, even for >1h. Hence, the Saos-2 cells were seeded

at a higher density: 1.5 x 10^6 cells/100 mm dish (i.e. ~ 2.5 x 10^4 cells/cm^2) into 100 mm dishes rather than 75 cm^2 flasks, and harvested by scraping.

On Day 1: CM mixes were prepared as solutions comprising 2% FBS, 48% serum-free DMEM and 50% [CM +/- serum-free T-medium], with the final concentration of CM at 0, 5, 10, 25 and 50%.

The FBS concentration in these experiments was reduced from the standard 10% to 2% in order to "encourage differentiation rather than proliferation" and to minimize the impact of early response genes on regulating ALP, OCN, COL, OPG and RANKL expression.

Cells were treated with 10 mL medium containing the various concentrations of CM, and 1 flask/dish of each CM concentration was harvested 3, 6 and 12h after initial exposure (5 flasks/dishes per timepoint). One mL of each culture supernatant (SN) was stored for analysis of secreted factors (-20°C); the remaining SN was aspirated. The cells were washed once in 5 mL PBS, then harvested by either trypsinization or scraping. The cell suspensions were counted, centrifuged, and the cell pellets homogenized in TRI Reagent (Sigma; Cat. No. T-9424) as per the manufacturer's instructions. These samples were stored at -80°C, until processed for the extraction of RNA and protein.

Day 2: The 24 hour timepoint flasks/dishes were harvested and processed as described above. The 48 hour timepoint cells were re-fed with 10 mL of the appropriate, freshly prepared CM solutions.

Day 3: The 48 hour timepoint flasks/dishes were harvested and processed as described above.

vi. Processing of samples: RNA was extracted from the TRI Reagent homogenates as per the manufacturer's instructions, and the samples stored at –80°C. Either immediately, or after samples had been stored overnight at 4°C, protein was also extracted from the aqueous phase of the homogenates, again as per the manufacturer's instructions.

vii Preparation of cDNA: To date, cDNA has been produced from the MG-63 and U-2 OS RNA samples. RNA concentration was determined using spectrophotometry, and each sample diluted to 1 µg/µL in RNase-free H_2O. Five µg/five µL RNA were diluted in 5 µL RNase-free H_2O, and 1 µL 100 µM random primers (Invitrogen; Cat. #48190-011) added. The mixture was incubated at 70°C for 5 min, then on ice for 5 min, followed by brief centrifugation. The following reagents were then added in the order given: 4 µL 5 x Reaction Buffer (Fermentas; supplied with the RevertAid, below), 2 µL 10 mM dNTPs (Fermentas; Cat. #R0181), 1 µL RNase inhibitor (Fermentas; Cat. #E00312) and 1 µL RNase-free H_2O. The mixture was incubated at room temperature (RT) for 5 min, then 1 µL RevertAid M-MuLV reverse transcriptase (Fermentas; Cat. #EP0442) added, and the mixture incubated for a further 10 min at RT, then at 42°C for 60 min, then at 70°C for 10 min. The cDNA was quenched on ice for 5 min, briefly centrifuged, and stored at –20°C.

Task 1B-a/Task 1C-b: Reverse transcriptase polymerase chain reaction

viii. Design of primers and optimization of RT-PCR conditions: Primers were designed and obtained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; positive control), alkaline phosphatase (ALP), osteocalcin (OCN), type I collagen (COL) and osteoprotegerin (OPG) (Table 1). It was intended to include in this study receptor activator of nuclear factor κ-B ligand (RANKL), a pro-resorptive factor in bone metabolism, but some difficulty was encountered with the designing of the primers; this section of the study will therefore be carried out at a later time.

Reverse transcriptase polymerase chain reaction (RT-PCR) conditions for these primers were initially optimised using *Taq* polymerase and MG-63 cDNA produced from RNA extracted from TRI Reagent homogenates, as described in the Annual Report, January, 2002, Task 1B-a/Task 1C-b, section vi, and Figures 2 and 3, Appendix 2. However, it was later determined that for some of the primer sets, a "hot-start" PCR protocol gave a better quality product. Additionally, it was necessary to optimize conditions for U-2 OS cDNA as well as for MG-63. Therefore, temperature and cycle number titrations were carried out with cDNA from both cell lines, comparing the use of a standard *Taq* protocol with one based upon Platinum® PCR SuperMix (Invitrogen; Cat. #11306-016), which had previously been shown to eliminate the problem of spurious band amplification with the OCN primers (discussed in Annual Report, January 2003). It was determined that U-2 OS cells expressed only minimal levels of OCN; these samples were subsequently omitted from analysis. The final PCR conditions are given in Tables 1 and 2, below:

Table 1: Design of primer sets

Primer set	Sequences	PCR System
GAPDH	Sense: 5'-CCA CCC ATg gCA AAT TCC ATg gCA-3' Antisense: 5'-TCT AgA Cgg CAg gTC Agg TCC ACC-3'	*Taq*
ALP	Sense: 5'-CCA gAg AAA gAg AAA gAC CCC AAg TA-3' Antisense: 5'-ATg CCC ACA gAT TTC CCA gCg TCC TT-3'	Platinum® PCR SuperMix
COL	Sense: 5'-AAg ACg ACA TCC CAC CAA TCA C-3' Antisense: 5'-AgC TTC CCC ATC ATC TCC ATT CTT T-3'	*Taq*
OCN	Sense: 5'-CCC TCA CAC TCC TCg CCC TAT Tgg CCC-3' Antisense: 5'-ggg CAA ggg gAA gAg gAA AgA Agg gTg C-3'	Platinum® PCR SuperMix
OPG	Sense: 5'-gCC CCT TgC CCT gAC CAC TAC TAC AC-3' Antisense: 5'-gCT gTg TTg CCT TTT ATC CTC TCT A-3'	Platinum® PCR SuperMix

Table 2: PCR conditions

Primer set	$MgCl_2$ concentration	µl cDNA	Regime	Cell line/ Cycle number
GAPDH	1.5 mM	1.0	94°C 4 min; 94°C 30 sec, 60°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 20 cycles U-2 OS: 20 cycles
ALP	N/A	1.0	94°C 4 min; 94°C 30 sec, 65.4°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 32 cycles U-2 OS: 35 cycles
COL	1.5 mM	1.0	94°C 4 min; 94°C 30 sec, 61.5°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 22 cycles U-2 OS: 22 cycles
OCN	N/A	1.0	94°C 4 min; 94°C 30 sec, 71°C 20 sec, 72°C 30 sec	MG-63: 35 cycles U-2 OS: N/A
OPG	N/A	2.0	94°C 4 min; 94°C 30 sec, 60°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 30 cycles U-2 OS: 33 cycles

ix. Analysis of experimental samples: Under the conditions given above, PCR was carried out on the MG-63 and U-2 OS experimental samples. For each set of five timepoint samples, the 0, 25 and 50% CM-treated samples were analysed. The PCR products were stored at –20°C.

The MG-63 PCR products were further examined by agarose gel electrophoresis using gels ethidium bromide, and run in TAE buffer containing ethidium bromide to the same concentration. Five µL of a 25 µL PCR product were run against a GeneRuler 100 bp ladder prepared at 1.5% agarose in Tris-Acetic Acid-EDTA (TAE) buffer containing 3 µL 10 mg/mL (Fermentas; Cat. # SM0241) at 80 V for 45 min. Gels were analysed under UV illumination using the Electrophoresis Documentation and Analysis System 120 (EDAS; Kodak). Net band intensity in pixels was recorded for each sample. Results were expressed relative to the levels of GAPDH, which is a "house-keeping" gene. A result was considered significant if a two-fold change in expression levels in either direction was recorded.

Results:

To date in the analysis, which is still in progress, certain trends have been identified that will warrant further investigation. The 6 CM were found to have a range of effects upon the levels of gene expression in the MG-63 cells. The LNCaP-P CM was found to suppress the expression of the ALP, OCN and OPG genes by 15-30% after 12 h treatment. Levels returned to normal after 24 h for ALP and OPG, but remained suppressed for OCN. LNCaP-empty had a biphasic effect upon OCN, with a transitory stimulation of expression after 3 h followed by suppression to ~ 50% of base levels after 48 h. LNCaP-empty also exerted a suppressive effect upon ALP, which was ~ 65% of base levels after 48 h. The LNCaP-wtp53 CM also exerted suppressive effects upon ALP and OCN, reducing their expression after 48 h to ~75% and ~65% of base levels, respectively. LNCaP-wtp53 CM also reduced the expression of COL by ~20% after 48 h.

The effects of the CM from the LNCaP cells carrying *p53* mutations were varied. As with LNCaP-empty, LNCaP-F134L transitorily stimulated OCN, then suppressed expression to ~65% of base levels after 24 h, with suppression continuing, although less markedly, at 48 h. Conversely, LNCaP-F134L stimulated the expression of both COL and OPG, with COL levels raised by 30-50% (see Appendix 2, Figure 2) and OPG levels by 60-65%, after 48 h. The effects of LNCaP-M237L were largely suppressive, with OCN levels reduced to ~75% of base expression after 3 h. This effect persisted to 48 h, where expression was 55-65% of base. ALP, COL and OPG were also suppressed after 48 h, by 15-20% in each case. LNCaP-R273H, in contrast to the results observed for the proliferation assays (see above), had little effect upon the MG-63 cells.

Discussion:

The results obtained in these differentiation assays indicate that prostate cancer cells carrying certain *p53* mutations could potentially exert significant effects upon bone metabolism following the establishment of a metastatic deposit. The factors investigated in this study are fundamental components of the osteoblastic differentiation pathway, and involved in the normal regulation of bone. Disruption of their expression could have profound effects upon bone metabolism. For example, simultaneous suppression of OCN, a negative regulator, and stimulation of COL, as observed after treatment with F134L CM, could play a role in the frequently observed accumulation of woven bone following prostate cancer metastasis.

Before such conclusions can be drawn, we need to ensure that the suppressive effects observed represent true alterations in cellular differentiation, and are not the result of toxicity. These experiments are currently underway. Similarly, the stimulatory effects exerted by the CM will be investigated using antibodies to potential factors to block the action of the CM.

TASK 1B-b, -c, and -d. Quantification of ALP, OCN, COL and OPG levels secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

TASK 1C-d. Assay for carboxyterminal propeptide of type I collagen using the Prolagen-C kit secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

x. Protein assays: The following kits were obtained in order to analyse the effects of the LNCaP series CM upon the secretion of factors by the osteosarcoma cell lines:

(1) Metra™ *CICP EIA kit* (Quidel Corporation, San Diego, CA; Cat. # 8003) - for the detection of the C-terminal propeptide of type 1 collagen in serum/culture supernatant
(2) Intact human osteocalcin ELISA kit (Biomedical Technologies, Stoughton, MA; Cat. # BT-460)
(3) Osteoprotegerin enzyme immunoassay (Biomedica, Gesellschaft, mbH; Cat. # BI-20402)
(4) Alkaline phosphatase diagnostic kit (Sigma, St Louis, MO; Cat. # 104-LL)

In one case, a new company took over the local distribution of one of the required kits, but was unable to acquire the necessary AQIS (Australian Quarantine and Import Services) permits to import them for over five months. However, all kits have now been obtained, and analysis of the SN samples is underway.

Analysis of samples for ALP, however, ultimately required the development of new methodology. Optimization of the Sigma kit listed above indicated that its limits of detection were not low enough to be suitable for the analysis of our samples. A second commercial kit also obtained from Sigma, was subsequently taken off the market, and could not be used for the analysis, as one kit was not sufficient to analyse all of the samples. Given the availability of no other suitable commercial kits, (and unhelpful literature), we decided to construct our own method for sample analysis.

The method developed is based upon the fact that the standard experimental reaction of native ALP upon the commercial substrate, *p*-nitrophenyl phosphate (pNPP), generates a product, *p*-nitrophenol (pNP), which can be quantified by optical density (OD) readings at 405 nm. Moreover, this OD reading is directly proportional to the amount of pNP generated by the action of ALP – e.g. an OD of 1.0 may indicate 5.0 μmoles of pNP. The method is therefore carried against two standard curves, one constructed using known amounts of pNP, the other using known concentrations of ALP. The assay is flexible, allowing for different sample sizes, 20-80 μl, to be analyzed, according to the reactivity of the particular sample batch. Plate readings are taken at regular intervals, usually hourly, or as appropriate, and linear regression analysis of the ALP standard curve used to determine the timepoint at which the reaction is complete.

The protein concentration of the experimental samples is also assessed, using either the BCA Assay (Pierce Biotechnology; Cat. #23223, #23224 and #23209) or the Coomassie® Plus Protein Assay (Pierce Biotechnology; Cat. #23236). The overall results are then normalised, and expressed in terms of μmoles pNP/mg protein.

Using this method, analysis of the experimental samples is currently underway.

Task 1C-e. Identify factors involved in mediating differing effects of the cell lines on osteoblasts and osteoclasts:

xi. Microarray: All six cell lines were cultured for approximately three weeks and were simultaneously processed to produce mRNA. The mRNA was quantified by UV spectroscopy and a small sample was analysed by agarose gel electrophoresis to check quality. The human TranSignal™ p53 target gene array kits were obtained from Panomics. In collaboration with Dr Shaun O'Mara (Dept Clin Medicine, UNSW) the genes on these arrays were spotted in duplicate with columns to the right and bottom of each array containing double spots of biotinylated DNA for normalisation purposes. The analyses were performed according to manufacturer's instructions.

In the first instance, we analysed the gene expression pattern of LNCaP-empty versus that of LNCaP-F134L. The blots were treated and washed, then were exposed to the same film at the same time. Multiple exposures were taken to optimise exposure time. One set of these exposures is displayed in Appendix 2, Figure 3. Those genes whose expression was found to be altered between the LNCaP-empty and LNCaP-F134L are shown in Table 3.

The membranes have been stripped, checked, dried and are now ready for the next analysis. Once all 6 lines have been analysed, we will perform RT-PCR on 5-10 genes for confirmation. We will also aim to analyse protein expression levels using ELISAs and Westerns. We can then go on to inhibit the function of some of these proteins using available neutralising antibodies or inhibitors.

Table 3: Up or down-regulation of genes in LNCaP-F134L compared with LNCaP-empty

Location on blot	F134L expression relative to empty	Gene
A8	Decreased	Beta-actin
B1	Increased	BAG1, bcl-2-associated athanogene
B3	Increased	Bax
D9	Increased	PMAIP1, phorbol-12-myristate-13-acetate-induced protein-1
E1	Increased	GPX, glutathione peroxidase
E2	Increased	Hsp70, heat shock protein 70 kDa
E5	Decreased	IGF-R, insulin-like growth factor 1 receptor
E10	Increased	PPM1D, protein phosphatase 1D magnesium-dependent, delta isoform
F1	Increased	Killer/DR5
F9	Increased	PRG1
F10	Increased	Pro.Ox., p53 induced protein
G1	Increased	MMP1, matrix metalloproteinase 1
G10	Increased	PTGF, prostate differentiation factor (PLAB)
H9	Increased	PTHLH, parathyroid hormone-like hormone (also called PTH-related protein)
I6	Decreased	RNA polIII, RNA polymerase III (DNA-directed) polypeptide K
I8	Decreased	TGF-alpha
J7	Decreased	TRID/TRAIL-3, decoy receptor, no intracellular domain
K3	Decreased	UBIQ, ubiquitin
K8	Decreased	ADFP, adipose differentiation-related protein
K9	Decreased	Slac19A1, solute carrier family 19 (folate transporter), member 1
M1	Decreased	CART, cocaine- and amphetamine-regulated transcript
M4	Decreased	P14ARF, cyclin-dependent kinase inhibitor 2A
N7	Decreased	Hic-1,hypermethylated in cancer 1
N9	Decreased	Tyrkin, tyrosine kinase-related sequence
O2	Decreased	Hsp27, heat shock protein 27 kDa
O3	Decreased	IGFBP6, insulin-like growth factor binding protein 6
O4	Decreased	Jun, v-jun sarcoma virus 17 oncogene homolog
O5	Decreased	Ker15, keratin 15
O6	Decreased	LATS2, large tumour suppressor 2

Task 1D. Effects of LNCaP transfectants on mineralization of collagen

Objectives:

The overall aim was to investigate the effect of factors secreted by the six LNCaP cell lines on matrix mineralization by human osteoblastic cell lines, Saos-2 and U-2 OS. However, we have shown that U-2 OS cells do not produce mineralized matrix *in vitro*. MC3T3-E1 calvarial cells have been used in preference to Saos-2 cells, as they have been widely used to study *in vitro* matrix mineralization (Sudo *et al.*, 1983, Kodama *et al.*, 1986, Fratzl-Zelman *et al.*, 1998). Moreover, MC3T3-E1 cells were considered to be the most appropriate for use in our experiments as they are primary murine cells, reflecting the situation that is being examined *in vivo* (Task 3).

Methods:

xii. Visualization of mineralized extracellular matrix: Mineralized extracellular matrix is commonly visualized by either a von Kossa (Rungby *et al.*, 1993) or Alizarin red S (Hale *et al.*, 2000) staining of calcium phosphate deposits. The two methods of staining were compared using densitometry and Alizarin red S staining was established as the method of choice for these experiments. The staining

protocol was optimized for MC3T3-E1 cells as follows: The medium is removed and the cells are fixed in 10% neutral buffered formalin for 30 min, washed twice in distilled water for 2 and 15 min respectively, and then stained with 2% Alizarin red S for 1 minute, washed 3X with distilled water (2, 10 and 15 min, respectively) and air dried for analysis.

xiii. Further optimization of methods for studying matrix mineralization by MC3T3-E1 cells *in vitro:* In order to increase the deposition of extracellular matrix and to promote the formation of osteoid-like nodules consisting of thick layers of type 1 collagen (Fratzl-Zelman, *et al.*, 1998) the protocol for culture of MC3T3-E1 cells was further optimized as follows:

To obtain maximum matrix mineralization in a positive control culture, the following method has been devised. Mouse calvarial cells, MC3T3-E1 are seeded at 1 x 10^5 cells/ well in a 6 well plate in αMEM with 10% FBS (day 1). On day 3 the medium is changed to αMEM 5% FBS supplemented with ascorbic acid (AA) (50 μg/mL), which is essential for collagen deposition within the extracellular matrix. Although ascorbic acid is present in the αMEM, it is readily oxidised and has to be replenished with each medium change (Hughes and Aubin, 1998). The medium is also supplemented with 10^{-8} M dexamethasone, which we have observed to increase cell attachment in long-term cultures. The cells are cultured for 4 weeks with a medium change every 2-3 days. The long-term culture systems for osteoblastic cell lines under these conditions promote increased deposition of extracellular matrix and the formation of osteoid-like nodules (nodule-formation stage). To induce mineralization of the nodules, 10 mM β-glycerophosphate is added to the medium and the cells are cultured for a further two weeks (mineralization stage), with medium changes every 2-3 days. This treatment induces baseline mineralization of the secreted extracellular matrix (negative control), which can be assessed by densitometry following staining with either Alizarin red S or von Kossa. In order to increase mineralization of the extracellular matrix (positive control), the cells are treated with 10^{-8} M dexamethasone (dex) continuously. Following this incubation period the medium is removed, the cell layer washed twice with PBS and fixed in 10% neutral-buffered formalin for 30 min at room temperature, washed and stained for Alizarin red S as in part ix above. The results are analysed by densitometry using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Results:

We have shown that the addition of ascorbic acid to MC3T3-E1 cultures results in increased matrix mineralization (Appendix 2, Figure 3). As demonstrated in Appendix 2, Figure 4, Alizarin red S staining produces a higher densitometric signal compared with von Kossa stain.

Objective

To optimize the condition for use when osteoblastic cultures are treated with varying concentrations of conditioned medium from the six LNCaP lines.

xiv. Optimization of conditions for mineralization in the presence of T medium.
The LNCaP cells are grown in the T medium, based on Dulbecco's modified Eagle's medium (DMEM), but we have previously shown that DMEM does not adequately support MC3T3-E1 cell growth and as a result, cell death occurs. It was therefore necessary, prior to commencing experiments using CM, to establish whether the two weeks' exposure of MC3T3-E1 cells to 50% T medium would allow them to produce mineralized matrix.

Results

We have shown that MCT3T-E1 cells did not mineralize their extracellular matrix in the presence of 50% T medium (Appendix 2, Figure 5A). Furthermore, cell layers became very fragile and tended to detach from tissue culture plastic, in contrast to what has been reported last year. We have demonstrated, that when 10^{-8} M dex is introduced during the mineralization stage (the last two weeks of culture) the MC3T3-E1 cells can mineralize their matrix in the presence of 50% T medium. The extent of matrix mineralization under these conditions appears to be indistinguishable from that produced by positive control cultures as indicated by densitometric analysis (Appendix 2, Figure 5B). Given that it would be undesirable to expose the MC3T3-E1 cells to 10^{-8} M dex during treatment with CM, as there is a possibility that dex may mask or interfere with the effects of factors present in the CM, we have selected 25% T medium as the maximum concentration, since MC3T3-E1 cells can mineralise in the presence of 25% T medium without dex (Appendix 2, Figure 5C). We have also investigated the effect of dex treatment prior to the mineralization stage (that is, during the first four weeks of culture) on the stability of the MC3T3-E1 cells and their ability to mineralize the extracellular matrix in the presence of 25% T medium. As this was helpful, it has been adopted as our method of choice for the CM experiments.

Objective:
To investigate the effects of factors secreted by the six different LNCaP parent and transfectant lines on mineralization by MC3T3-E1 cells.

Methods

xv. Exposure of MC3T3-E1 cells to CM: The MC3T3-E1 cells were cultured for 4 weeks to develop nodule-formation as described above (part *xiii*) in αMEM plus dex (10^{-8} M), 5% FBS and ascorbic acid (50μg/mL). They were then treated with 5, 10 and 25% CM (prepared as in Method i and pooled) for 2 weeks in the presence of βglycerophosphate (10mM). Medium was changed 3 times/week, (total of 6 treatments), and the cells were then fixed and stained with Alizarin Red S (see *xii* above).

Results:

The results are expressed as a ratio of CM-treated cells compared with cells cultured in the presence of 25% T medium (Appendix 2, Figure 6). Experiments were performed twice. Only minor effects of the CM were generally seen, with the exception of that from F134L, where 25% CM caused inhibition of matrix mineralization. To confirm this effect, in future experiments the MC3T3-E1 cells will be co-cultured with LNCaP-F134L cells.

Task 1E. Effects of CM from LNCaP parent and transfected lines on mouse osteoclast differentiation *in vitro*.

Objective:
The aim was to investigate the effect of factors secreted by the six LNCaP cell lines (LNCaP-parent (LNCaP-P), Empty, and those expressing either wtp3 or mutant p53, i.e., F134L, M237L, R273H) on osteoclastic bone resorption.

Methods:

xvi. Effects of CM on osteoclastogenesis (collaboration with Julian M. W. Quinn from St. Vincent's Institute of Medical Research, Victoria, Australia).

Osteoclast differentiation from precursor cells has been reported to require stimulation with two factors: receptor activator of nuclear factor-κB ligand (RANKL) (Hofbauer and Heufelder, 2001) and colony-stimulating factor-1 (CSF-1) (Scheven *et al.*, 1997). In these reported studies, stimulation of mouse bone marrow cultures with 30 ng/ml RANKL and 25 ng/ml M-CSF for 3-4 weeks resulted in the appearance of cells staining positively for tartrate-resistant acid phosphatase (TART), indicating the presence of osteoclasts.

The femurs of 4-8 week-old C57Bl/6J mice were harvested and the bone marrow was flushed from the femoral cavity using PBS. The cells were plated in quadruplicate into 96-well plates at 3.2×10^4 cells/well in αMEM with 10% foetal bovine serum (FBS), 100 ng/ml RANK ligand (RANKL), 25 ng/mL CSF-1 and 25% CMs or serum-free T-medium (vehicle control). Media plus supplements were replaced on day 3. Negative control wells contained cells that were treated in the absence of RANKL. Cells treated with αMEM and 10% FBS, 100 ng/mL RANKL and 25 ng/mL CSF-1 were used as positive controls. Resultant cells were stained for stained for TART and counted.

Results:

The results are shown in Appendix 2, Figure 7. When compared with T-medium, CM from LNCaP-Parental (LNCaP-P) cells significantly inhibited osteoclastogenesis. When compared with CM from the empty transfectants, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation. When compared with CM from wtp53 transfectants, that from F134L was significantly anti-osteoclastogenic.

Discussion

We found that LNCaP-P cells secrete factors that inhibit osteoclast formation. Empty control cells stimulated osteoclastogenesis over parental cells, suggesting that transfection affected the ability of the cells to modulate osteoclast formation. When compared with empty controls, wtp53 and R273H had no effect on osteoclast formation, whereas the F134L and M237L mutants inhibited osteoclastogenesis, suggesting that these p53 mutations modulate the secretion of factors that influence osteoclast formation.

The changes in osteoclastogenic activity do not occur through increased OPG protein levels, as determined by ELISA (data not shown), and it is unlikely that soluble RANKL plays a role since treatment with CMs in the absence of exogenous RANKL produced no osteoclasts. The most likely explanation, therefore, is that the effects of the CMs on osteoclastogenesis are mediated indirectly through mouse bone marrow stem cells present in the primary culture.

TASK 2A Effects of LNCaP transfectants on endothelial cell proliferation *in vitro.*

Objective:

The human endothelial HUV-EC-C cells were treated with CM produced by the six LNCaP cell lines to determine whether there was any effect on cell proliferation. Proliferative effects of CM were also assessed on an immortalised bone marrow endothelial cell line, BMhTERT cells (Wen et al., 2003), obtained through collaboration with Dr Karen McKenzie, Children's Cancer Institute of Australia. Since prostate cancer cells spread to the bone, and as endothelial cells are specific to the organ from which they are derived, we anticipated that the CM from the LNCaP tranfectants would elicit a significant response in human bone marrow endothelial cells compared with human umbilical vein endothelial cells.

Methods:

The following were completed and described in DOD report, Jan, 2003 (Task 1A method i):
i Production of CM: This was done as described above (Task 1A, method i).

xvii Creating stocks of the HUV-EC-C cells: (see Annual report, Jan 2003). In brief, 30 ampoules of HUV-EC-C (CRL-1730, ATCC, passage 15) cells were derived (passages 16-23) and stored ($5x10^5$-$1x10^6$ cells/ampoule).

xviii. Determination of optimal seeding for HUV-EC-C cells: (see Annual report, Jan 2003). The appropriate seeding numbers for HUV-EC-C cells in fresh F12K medium, 10% FBS, with additives (HUV-EC-C medium, Appendix 1) were determined to ensure that they were in log phase after 1 week in culture by carrying out cell density experiments. Using the WST-1 Cell Proliferation Assay (Roche; Cat# 1 644 807), the optimal cell density for seeding was found to be 1000 cells per well.

TASK 2A-c
xix. Optimization of medium for proliferative experiments: As CM contains T medium without FBS, whereas HUV-EC-C cells are grown in F12K and additives (HUV-EC-C medium, Appendix 1), and BMhTERT cells in EBM-2 medium, Appendix II) it was necessary to incubate the endothelial cells in a 50 % mixture of T medium and their specified media to determine their survival as well as the optimal percentage of FBS for their growth. Endothelial cells were plated in triplicate in 96 well plates, HUV-EC-C cells at seeding densities of $5x10^2$ to $4x10^3$ cells per well in four sets and BMhTERT cells at 250 to 8000 cells per well in a gelatinised 96 well plate Each set of seeding densities was maintained in different medium mixtures as follows:
HUV-EC-C cells:
1) HUV-EC-C medium, 10% FBS
2) 45% HUV-EC-C medium + 45% T medium (Mixed medium) +10% FBS
3) Mixed medium + 5% FBS
4) Mixed medium + 2% FBS

The monitoring and analysis of this experiment were done as described in method xviii, except that the standards were plated at 0, 250, and doubling concentrations thereon to 128000 cells/well.

Results:

HUV-EC-C cells: The optimal seeding cell density in the mixed medium with 5% FBS was also established for HUV-EC-C cells at 1000 cells per well (see Annual Report, Jan 2003). The cell proliferation obtained in mixed medium with 5% FBS was similar to that seen in HUV-EC-C media, with 10% FBS.

BMhTERT cells: The initial seeding densities were established in the standard BMhTERT media (EBM-2 media, Appendix 1). The cells were fed with either 100% EBM-2 or 50% EBM-2 media plus 50% T-Media without FBS (mixed medium) on days 1 and 4 after seeding and harvested on the day 6, with WST-1 reagent. The optimal seeding density in the mixed medium was 2000 cells per well over 6 days, but a reduction in the proliferative rate of the BMhTERT cells was seen in mixed medium compared with that in EBM-2 medium (Appendix 2, Figure 8). At lower seeding densities (i.e. 250, 500) cells in mixed medium detached and died while those at the same densities in 100% EBM-2 media proliferated. This suggests that the higher concentration of growth factors in the standard endothelial medium compensated for the lack of cell to cell contact at lower seeding densities whereas this did not occur when EBM-2 was mixed 50:50 with T medium.

xx. Effects of CM on proliferation of endothelial cells

Methods:

HUV-EC-C cells: Different percentages of CM (0, 5, 10, 25 and 50%) from each of the 6 LNCaP cell lines were prepared by diluting the appropriate volume of CM to 50% of the final volume in T-medium without FBS and adding an equal volume of HUV-EC-C media (50%). The effects of the CM on HUV-EC-C cell proliferation were assessed on triplicate cultures of HUV-EC-C cells, seeded on day 0 at 1000 cells per well in 96 well plates, and microscopically examined for cell density on days 2 and 5, when CM mixtures were exchanged. On day 7, the cells were observed and the media were replaced with phenol red free DMEM. To provide a standard curve, serial dilutions of untreated HUV-EC-C cells in phenol red free DMEM were plated in triplicate at doses from 500 to 64,000 cells/well, with triplicate "medium only" wells being used as negative controls to provide the degree of background optical density on each plate. After adding 10µl of the WST-1 Cell Proliferation Assay (Roche; Cat# 1 644 807) reagent to each well, the optical density was read after 0.5, 1, 2 and 4 hours at 450nm (Tecan, Sunrise plate reader, Tecan, Salzburg, Austria). The R^2 value was calculated to determine the incubation time showing the best linearity over the standards, and the cell numbers were compared using one-way ANOVA and Tukey's post-tests (Graph Pad Prism). For each type of CM a ratio of the absorbance at 0% to that at 5, 10, 25 and 50% CM was determined. The ratios were compared using a One Way ANOVA and Dunnett post-test. Dose responses to the different CM treatments were compared.

BMhTERT cells: The effects of CM from LNCaP-Empty and LNCaP-R273H on BMhTERT cells have also been ascertained. The experimental design was similar to that for the HUV-EC-C cells described above except that cells were seeded in EBM-2 medium, were fed on days 1 and 3 and harvested after 6 days. Doses of CM used were 6.25, 12.5, 25 and 50 %. The CM used in these experiments were prepared by a standardised production protocol (method ii) to allow direct comparison of the effects of CM from different cell lines. For the standard curve, BMhTERT cells were plated in triplicate, at 32000, 16000, 8000, 4000, 2000, 1000, 500 and 0 cells/well. The analysis was performed as for the HUV-EC-C cell line. The dose responses to CM from LNCaP-Empty and LNCaP-R273H were compared.

Results:

HUV-EC-C cells: The CM of the LNCaP-F134L at 10, 25 and 50% was found to inhibit the proliferation of HUV-EC-C cells significantly (Figure 9). Trends suggestive of inhibition were also seen at the higher doses of CM from the parental and LNCaP-R237H lines.

BMhTERT cells: The 12.5, 25 and 50% CM of the LNCaP-Empty and LNCaP-R273H were found to inhibit BMhTERT cell proliferation significantly (Figure 10). Currently, we have not ascertained whether this inhibition of proliferation is due to an increase in cell differentiation or apoptosis. The effects of the other CMs on BMhTERT cell proliferation will be completed in early 2004.

TASK 2 B. Effects of LNCaP transfectants on endothelial cell differentiation *in vitro*

Objective:

The human endothelial cell line, HUV-EC-C, was treated with CM produced by the six LNCaP cell lines (parent, Empty, and those over expressing Wt or expressing mutant p53) to determine whether there is any effect on cell differentiation.

Method:

xxi. Optimizing conditions for endothelial cell tube formation in matrigel. Frozen Matrigel was left to liquefy at 4°C for 2-3 hours, mixed 1:1 with EBM-2 media to provide matrix mix, and added at 60 µL/well before incubating the plate for 30min at 37°C to polymerise the matrix. BMhTERT cells were harvested, counted and resuspended at: 5000, 10,000 and 20,000cells/200 µL. Then 200 µl of each cell suspension was added to triplicate wells coated in matrix mix. Two different media mixes, 100% EBM-2 and 50% EBM-2 plus 50% T-media without FBS, were used. Photographs of all wells were taken 0, 4, 16 and 24h later and their analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Incubation with crystal violet (0.5%) for 20 mins creates increased contrast enabling easier analysis. Averages of triplicate wells will be used to compare the tube length, tube width and number of branching points. This work is in progress.

Results
Tubes were observed to form in both EBM-2 and mixed medium when seeded at 20,000 cells per well (Appendix 2, Figure 11). The cells had begun to migrate into alignment by 4h; by 16h, tubes had been formed, and these were more established by 24h. The effects of other CM treatments on HUV-EC-C cell differentiation should be completed in early, 2004.

TASK 3A Role of p53 in bone metastasis *in vivo* using the osseous-CaP bone injection model
Initial Take Rate Study
Objective:

To determine the take rates of the 6 LNCaP cell lines when injected into the tibia of Severe Combined Immunodeficient (SCID) mice. This was accomplished by assaying the host sera for prostate specific antigen (PSA), by X-ray analysis (Faxitron analysis) of the mice and by examination of paraffin embedded tissue from the mice stained with hemotoxylin and eosin (H&E).

Methods:
xxii. Intra-tibial injections and tumor growth: Methodology was described in the January, 2003 report in Section xvi.
In brief, 6-8 week old SCID mice were anesthetized singly by inhalation anesthetic, Isoflurane (5%), carried in oxygen in an induction chamber, and anesthesia was maintained throughout by using a face mask (around 2 minutes). The ankle joint of the left leg of a mouse on its back with its head towards the injecting hand was immobilized between the thumb and forefinger of the left hand (if right handed) and positioned with the knee flexed. Cells for injection (20µL at 2-4x10^7cells/mL) in a 1mL syringe with a 26G needle were injected directly into the proximal epiphysis of the tibia with gentle, unforced drilling, and the needle was removed by the same process in reverse. Analgesia was provided by an intraperitoneal injection of Buprenorphine, (Temgesic, Reskitt and Coleman) at 0.01 mg/kg in a 100µL volume. The mouse was removed from the anesthetic line and allowed to recover.

The mice were monitored daily. They were weighed and their tibias palpated for tumors twice weekly (according to animal ethics from the Animal Care and Ethics Committee, UNSW, ACEC# 01/102). Every 3-4 weeks, mice injected with LNCaP tumor cell lines were bled for PSA testing. Between 50-100µL serum was diluted with Universal diluent (Roche, Cat #1732277) to 350µL and assayed for PSA using a PSA kit (Roche, Cat #1731262). Eighteen weeks post injection, the mice

were sacrificed and X-rayed using a Faxitron (150 VAMAX, Model #MX-20, Auburn, CA, USA; settings 50sec and 20kv). Those mice that scored positive for a leg tumor by autoradiograph were determined to have a final serum PSA level of at least 1.2 ng/mL.

Experimental progress, 2003:

xxiii. Use of Faxitron to monitor intratibial tumor growth: In mid June, 2003, we purchased a Digital Faxitron (Micro focus system, Model# MX-20, Wheeling, Illinois, USA; settings 32 kv for 5000 msec) that has been set up within the animal facility. Experimental mice are serially X-rayed after anesthesia with Ketamine/Xylazine at 4, 8, 12, 16 and 18 weeks after intratibial inoculation of cells. Periodic tail bleeds are performed at the same time points. Mice are kept under sterile conditions by placing them in an autoclaved tip box for transfer from the biohazard hood to the Faxitron. This procedure does not interfere with the imaging. Each mouse is X-rayed 3 times; a comparative X-ray (1.5x, shelf 5) allows both hind limbs to be visualized at once and then a close up (3x, shelf 3) on the injected and then the control leg is performed, allowing more details to be observed. The Faxitron settings have been optimized for tibial observations.

Methods were established for tibia dissection for histological studies, fixation and decalcification of the samples, H & E staining, and PSA immunohistochemical staining of tissues and were described in last year's annual report (January, 2003).

Histology of bone:

xxiv. Tibia dissection: A longitudinal incision was made into the skin along the lower half of the hind leg. The tendons were cut just above the ankle, allowing the large muscle to be peeled from the bone. The bone was cut just above the knee joint (distal end of the femur) and also at the distal end of the tibia to allow the fixative to penetrate the bone.

xxv. Fixation and decalcification: The legs were placed in 10% Neutral Buffered Formalin for 24-48 hours, then washed in distilled water (dH_2O) before placing in 10% formic acid (15-20ml) in a Falcon tube. The tube was then placed on a rotating platform at an angle (ensuring even decalcification) overnight. The next day, a sample of the formic acid was removed to test for calcium deposits: 3 mL of the formic acid bathing the bone were removed and placed into a fresh tube together with 3mL of saturated ammonium oxalate to determine whether a precipitate forms (this takes about 10 minutes). If a white precipitate is present, the sample requires further decalcification. In this event, the remaining formic acid is removed from the sample and replenished with fresh formic acid. This was repeated daily until no precipitate formed with ammonium oxalate. The sample was then washed 3 times in dH_2O for at least 10min each wash to ensure that all formic acid is removed before placing the samples within the embedding machine.

xxvi. Optimization of decalcification and staining. We have now optimized the decalcification procedure to limit antigen destruction. Tibiae harvested from experimental mice were fixed for 24h at 4°C in 10% Neutral Buffered Formalin, then placed in 10% w/v EDTA disodium and rotated for 7 days at room temperature, before processing for paraffin embedding. The 7-day time point of decalcification was determined by daily X-ray (using the Faxitron) to compare tibia in EDTA with a tibia that had been previously decalcified using formic acid. We found that as the calcium was removed from the bone the density decreased. On Day 7, the density of the bone was similar to that of muscle and the X-rays of the tibia decalcified in EDTA or in formic acid were comparable. Following decalcification, the tissue was paraffin-embedded under standard conditions and stained with hematoxylin and eosin (H & E).

xvii. Immunohistochemistry for PSA staining: This was described in Annual Report, January, 2003.

xxviii. Preparation of paraffin embedded cell clots: To further characterise the LNCaP transfected cell lines paraffin embedded cell clots have been produced in duplicate for each cell line. Cells of each line were grown in 150 cm^2 flasks until 70-80% confluent, harvested using trypsin, counted and diluted to 2,000,000 cells/mL of media. The cells were pelleted and suspended in 80 µL of plasma before adding 20 µL of thrombin, which set rapidly. The clots were then fixed in 10% neutral buffered formalin for 24 hours, and embedded in paraffin by standard methods.

Results

In 2002, three groups of 10 SCID mice were injected intra-tibially with LNCaP, Empty or PC-3 (that are p53 null) cells to determine the take rate of the tumors. This was around 50-60% take rate for LNCaP-parental cells, and 80-90% for LNCaP-Empty, as measured by serum PSA levels or X-ray analysis, similar to that described in other studies (Wu *et al* , 1998). LNCaP cells formed mixed/osteoblastic lesions in the bone that were PSA positive (see Annual Report, January 2003). All but 2 of 40 mice were healthy throughout the experiments.

Given these promising results, 100 SCID mice received intratibial injections between mid March and the end of May, 2003. Initially, the mice appeared healthy during standard monitoring procedures but in mid to late May, a progressive loss of weight occurred in 6 mice. Each mouse was housed separately, euthanazed when they lost > 20 % of their peak weight (Appendix 3, 11) and a post mortem performed. Dr David Pass, who had supplied the mice (from ARC, Perth, Australia) was contacted when more mice began to lose condition, and he mentioned previous kidney problems in ARC SCID mice. He had thought that these problems had been resolved through re-derivation of the mice. Experimental and control mice in our laboratory sacrificed subsequently had small, pale or blotchy kidneys. Many mice lost > 20 % of their peak weight by 15 weeks of age (8 experimental weeks). As the injections were staggered over a 12-week period there was a progressive loss of mice rather than all at the one time.

As the mice aged they became increasingly difficult to bleed and the blood was dark and viscous. In addition, the mice appeared dehydrated and tended to form sores (after tail bleeding or fighting) easily that did not heal in the normal manner. Only 21% of the mice survived until the end of the 18-week experiment and these were not healthy. Upon dissection they were found to have pale and/or blotchy kidneys. All mice had their sera tested for PSA but none achieved the minimum value set last year (1.2ng/mL) to define an established tumor. A number of samples were cut and stained with H&E but no tumors were found.

Tissues from an experimental and a control mouse were analysed by a "mouse pathologist," Dr Malcolm France, Sydney University, including the head, heart, lungs, kidneys, gut, small and large intestine, bladder, spleen, liver and reproductive systems. He reported necrosis within the renal papilla (See Appendix 3). H&E slides of the major organs were also examined by Dr Jonathan Erlich (Renal Research Department, Prince of Wales Hospital) who found renal tubular infarcts and some evidence of myocarditis (see report in Appendix 3).

Given the poor blood supply due to the infarcted kidneys, angiogenesis may have been insufficient to allow tumour formation. Hence the above experiments need to be repeated. To find appropriate host mice, we are currently testing SCID mice from a different source (Walter and Eliza Hall Institute, WEHI, Melbourne, Victoria, Australia), Non-Obese Diabetic Severe Combined Immunodeficient (NOD-SCID) mice (also from WEHI) and Recombinant activation gene 1

(RAG1) mice from the ARC, Perth. Ten of each have been injected i.t. with control tumor cells (LNCaP-Empty) and 2 mice per species are being kept as age matched controls. Injected mice are being monitored for serum PSA, and by Faxitron analysis under anesthesia. These are currently at experimental week 8. Once tumors grow, we will select the appropriate strain of mice for repetition of our current experiments.

TASK 3B (months 3-18): Intratibial study

Methods:

xxix. mRNA extraction from bone: Optimization of the methodology was performed in 2002. We found that injecting Tri-Reagent into the bone marrow space provided a greater RNA yield with minimal degradation of the sample (Annual Report, January 2003, Appendix 7, Figure 9). As we now have a Faxitron in the animal house, this will allow us to perform X-rays prior to euthanasia of the mice and should facilitate the harvest of tumors for mRNA analysis.

Intra-tibial injections of the cell lines (LNCaP-P, LNCaP-empty, wtp53, F134L, M237L, R237H) were to have been completed by the end of May 2003 to provide tissues for analysis. However, as described above, problems with the host mice have delayed this work, which will have to be repeated once a suitable host strain has been obtained.

TASK 3c (months 8-15) Histomorphometric and immunohistochemical analysis of bones from Tasks 3a and 3b

Objective:

To perform histomorphometric analysis of intratibial tumors and bones

Methods:

xxx. Optimization of fixation for successful histomorphometry. Early in 2003, tibias injected with the human prostate cancer cell line PC-3, which is null for p53, were sent to Skeletech (WA, USA) for histomorphometric analysis. *Our protocol for fixation was shown to be successful.* The following is a section of their report:

"These bones were processed without decalcification and embedded in methyl methacrylate. The embedded tibias were sectioned parasagittally at 4 μm, two sections per bone. One section was stained for tartrate-resistant acid phosphate TRAP activity and counterstained with methyl green (nuclear stain) and thionin (cytoplasmic and metachromatic stain) for cell morphology, and the other section was stained with the Goldner's trichrome technique to help investigate the bone changes associated with tumor growth from locally injected tumor cells.

Static bone histomorphometry was performed on TRAP-stained sections in two separate regions of interest (ROI). These two ROI's were assigned for the following reasons: 1) The first ROI (in a zone area 0.2-6.2 mm distal to the growth plate) encompassed most of the proximal half of the tibia for the purpose of examining overall tumor growth as well as cancellous and cortical bone, and 2). The second ROI (in a zone area 3.2-4.1 mm distal to the growth plate) was chosen to examine the cellular activity in area where cortices were not completely removed by tumor-associated bone destruction. Tumor volume, cancellous bone volume and cortical bone volume in the first ROI was measured at 20X magnification. Cellular perimeter, which included osteoblast perimeter, osteoclast

perimeter and osteoclast number, in the second ROI were measured at 200X magnification. In addition to bone histomorphometry at the two ROI's described, the growth plate width was also measured at 200X magnification by tracing the growth plate area to obtain the average width, as calculated by the OsteoMeasure software program used.

It was found that all of the tibias that received injections of PC-3 cells showed high levels of tumor-related destruction of the cortical and cancellous bone. The degree of the tumour related destruction at the growth plate was more variable, with only one tibia not experiencing metastasis of the epiphysis. The tumor-bearing legs displayed extensive bone destruction, which appeared to be a sole osteolytic process and not accompanied by any distinctive osteoblastic reaction, i.e., increased new woven bone formation, while the contralateral controls showed no sign of tumor related destruction of the bone."

xxxi. Triple staining for p53, PSA and CD31 (endothelial marker) Our collaborator, Mr Kim Ow, Oncology Research Centre, Prince of Wales Hospital has developed a protocol for p53, PSA and CD31 triple staining of mouse bony tissues. This utilizes sequential enzyme/chromogen staining with indirect immunoperoxidase and phosphatase techniques using the Dako Envision Doublestain system (K1395) with some modification for triple staining.

1. The sections were prepared: Briefly, 5μm paraffin-embedded tissue sections were dewaxed in Histochoice (Amersco), rehydrated in a graded series of ethanol to water, immersed and boiled in 0.01M citrate pH6 solution for 10 mins in a 1000W microwave oven (maximum power), followed by 15 minutes cooling at room temperature (RT) and 5 mins washing in water. Non-specific mouse IgG binding was blocked using Vector M.O.M. kit (PK-2200). Endogenous peroxidase was quenched with Dako Peroxidase Block for 5 mins and rinsed with water.
2. The sections were serially incubated starting with the first primary antibody, mouse monoclonal anti-human p53-DO1 (Santa Cruz), diluted 1:40 in 1% BSA-TBS, overnight at 4^0C, rinsed in TBS/0.01%Tween20, then incubated for 30 mins with Dako Envision polymer-HRP followed by rinsing in TBS/0.01%Tween20. Brown colour was developed using Dako Liquid DAB (diaminobenzidine) for 5 mins, stopped by washing in water. A Doublestain Block (Dako) was applied for 3 mins and rinsed in TBS/0.01%Tween20.
3. The same steps were used for the second primary antibody, rabbit anti-human PSA (Dako) diluted 1:400 in 1% BSA-TBS, 1h at RT, rinsed in TBS/0.01%Tween20, but Dako Envision polymer-AP was used instead of polymer-HRP. Red colour was visualised using Dako Fast Red (5 mins) then washed in water. The Doublestain Block was applied for 3 mins and rinsed in TBS/0.01%Tween20.
4. These steps were repeated with the third primary antibody, rabbit anti-mouse CD31 (Santa Cruz sc-8306) diluted 1:200 in 1% BSA-TBS for 1 h at RT, rinsed in TBS/0.01%Tween20, then incubated for 30 mins with Envision polymer-AP followed by rinsing in TBS/0.01%Tween20. Magenta colour was developed using Dako Fuschin (5 mins) and thoroughly washing in water. The slides were lightly counterstained in methyl green (nuclei), washed in water and cover slipped in Dako Faramount aqueous mounting medium.

Results:
p53 (nuclear) and PSA (cytoplasmic) showed strong positive immunostaining in some cells but CD31 immunostaining could not be detected due partly to decalcification treatment of the tibia.

Task 4 (months 13-30): Metastatic capability of LNCaP transfectant cells implanted orthotopically.

Objective:

To investigate the metastatic capability of LNCaP cell lines. We had planned to inject the cells orthotopically, but reports (Bologna et al., 2002; Padalecki et al., 2003) indicate that intracardiac injection often allows cancer cells to spread to several organs. We believe that this methodology may give superior data compared with orthotopic injection.

Methods:

xxxii. Intracardiac injection: We have mastered this technique using a prostate cancer cell line that expresses green fluorescent protein (for ease of detection of metastatic deposits). Using PC3-hiEGFP cells (prepared by Dr JM Blair), 1 x 10^6 cells in 100μL were successfully injected into the left ventricle of the heart of 8/10 anesthetized mice. The experiment is to be conducted over a 4 month period. To date, mice have been followed by observation and palpation.

Results:
Preliminary data have indicated that one mouse has already developed tumors in the liver, lungs, and in the mandible 7 weeks post-injection of cells. This indicates that the technique for injection has been mastered and that this route of injection can lead to bony metastases. Intracardiac injection will be the preferred method of study for our LNCaP line and these studies will be undertaken during this year.

KEY RESEARCH ACCOMPLISHMENTS

- *Shown that LNCaP cells over-expressing p53 variants affect the proliferation of osteoblast-like cells in vitro.* The effects of CM from the six LNCaP lines tested on the proliferation of three osteosarcoma cell lines, MG-63, U-2 OS, and Saos-2, used as models of early-, mid- and late stage differentiation *in vitro* were typically biphasic for each osteoblast cell line. M237L CM stimulated the proliferation of U-2 OS and Saos-2 cells, whereas R273H was mitogenic for MG-63 and U-2 OS cells, suggesting differentiation stage dependent effects of these CMs. F134L and empty control CMs had little effect.

- Designed and sequenced primers and optimized RT-PCR for the expression of bone differentiation markers including alkaline phosphatase (ALP), osteocalcin (OCN) and type I collagen (COL) mRNA for examining the effects of CM from the 6 LNCaP cell lines on osteoblast differentiation *in vitro*.

- Incubated 3 osteosarcoma cell lines with CM from the 6 LNCaP cell lines at different doses and time points, and harvested 1350 samples each of cell homogenate in TRI Reagent, and of culture supernatant for RT-PCR and protein analysis.

- Based on studies of mRNA from osteosarcoma lines treated with CM from the 6LcaP cell lines, have shown that certain *p53* mutations could potentially exert significant effects upon bone metabolism following the establishment of a metastatic deposit. The factors investigated in this study are fundamental components of the osteoblastic differentiation pathway, and involved in the normal regulation of bone. Disruption of their expression could profoundly affect bone metabolism. For example, simultaneous suppression of OCN, a negative regulator, and stimulation of COL, as observed after treatment with F134L CM, could play a role in the frequently observed accumulation of woven bone following prostate cancer metastasis.

- Optimized a method for studying matrix mineralization by MC3T3-E1 cells *in vitro.* Shown that, as opposed to U-2 OS cells, the Saos-2 cells can produce mineralized matrix *in vitro.*

- Shown that CM from F134L inhibits matrix mineralization by MC3T3-E1, whereas CMs from other LNCaP- lines do not appear to have an effect.

- *Shown that LNCaP cells over-expressing p53 variants modulate osteoclastogenesis in vitro.* LNCaP-P cells secrete factors that inhibit osteoclast formation. When compared with T-medium, CM from LNCaP-Parental (LNCaP-P) cells significantly inhibited osteoclastogenesis. When compared with CM from the empty transfectants, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation. When compared with CM from wtp53 transfectants, that from F134L was significantly anti-osteoclastogenic. Thus, these p53 mutations modulate the secretion of factors that influence osteoclast formation.

- *Shown that the gene expression pattern of LNCaP-F134L mutant differs from the LNCaP-empty control.* The expression of several genes associated with metastasis, such as matrix metalloproteinase 1 and parathyroid hormone-related protein, and others associated with apoptosis, including Bax, DR5, hsp70 and BAG1, was modulated in the F134L mutant when compared with the LNCaP-empty control.

- *Shown that LNCaP cells over-expressing p53 variants modulate endothelial proliferation in vitro.* The CM of the LNCaP-F134L was found to inhibit the proliferation of HUV-EC-C cells significantly. This has not yet been tested on BMhTERT cells. However, CM from both LNCaP-empty and LNCaP-273H cells both inhibited the proliferation of bone marrow derived immortalised endothelial cells, BMhTERT.

- Established the take rate of LNCaP and Empty cells for forming tumors in the tibia of SCID mice; optimized histological techniques for their examination and immunohistochemical analysis and collected samples for bone histomorphometry.

- Using intratibial tumors of PC-3 (p53 null) prostate cancer cells for control studies, have shown that our fixation methods are appropriate for histomorphometric assessment.

- Established the technique for intracardiac injection for studies of spontaneous metastases by the LNCaP cell lines.

REPORTABLE OUTCOMES

Dr Blair (nee Brown) has presented a poster, entitled: Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells, by Brown JM, Szymanska B, Quinn JMW, Kingsley EA, Perryman LA, O'Mara SM, Jackson P, and Russell PJ, at the The IVth International Conference on Cancer-Induced Bone Diseases, Adam's Mark Hotel Riverwalk, San Antonio, Texas, Dec 7-9, 2003 (see Appendix 5).

CONCLUSIONS:

Our work to date has concentrated on the effects of factors secreted by LNCaP cell lines over-expressing wt or mutant p53s on bone and endothelial cells. It is possible that more pronounced effects will be observed when cell to cell contact experiments are performed. However, some conclusions of the effects of p53 mutations in the response of bone to factors secreted by CaP cells have been drawn, based on the above work. Taken together wtp53 had little effect on osteoclastogenesis but was mitogenic for maturing osteoblasts; F134L inhibited osteoclast formation but had little effect on osteoblasts; M237L inhibited osteoclasts but stimulated maturing and mature osteoblasts; and, R273H had little effect on osteoclasts but stimulated immature and maturing osteoblasts. Factors secreted by LNCaP-F134L cells but not by other LNCaP lines inhibited matrix mineralization by MC3T3-E1 mouse calvarial cells. In summary, these data suggest differing roles for p53 mutations in the response of bone to CaP cells.

Factors secreted by the LNCaP lines have also been shown to inhibit the proliferation of both HUV-EC-C and BMhTERT (bone marrow derived, immortalized) endothelial cells. The mechanisms by which this inhibition is achieved have not yet been determined.

The LNCaP lines have been shown to form tumors when implanted in the tibia of SCID mice, with take rates of ~60% for parental cells and ~80% for those containing the Empty cassette. However, due to illness in host mice, we have not been able to continue our *in vivo* studies. Once a new host model has been proven, we are in an excellent position to determine the role of *p53* mutations in the establishment and growth of bony metastasis from prostate cancer. These studies could lead to the demonstration of targets for new therapeutic strategies. We have mastered the technique for intracardial injection and shown that control cells spread to the bone when inoculated by this route. We will perform intracardial injections to follow the fate of LNCaP cells overexpressing wtp53 or mutants of p53.

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APPENDICES:

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Appendix 2: Figures 1-11
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Fig 2: Effects of LNCaP-derived conditioned medium on collagen production by osteosarcoma cells.
Fig 3: Microarray comparing LNCaP-empty and LNCaPF134L
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Appendix 3 Figure 1: Weight loss in SCID mice
Letter to D Pass, ARC
Reports from M France and J Sevastos

Appendix 4 Batches of LNCaP-derived conditioned medium (CM) and pooled CM used for Tasks 1D and 1E

Appendix 5 Poster

Appendix 1: Media ingredients

"HUV-EC-C- medium": 90% F12K, 10% fetal bovine serum and 30μg/ml of Endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO, USA) and 100μg/ml of Heparin (Sigma).

SOC medium: 2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

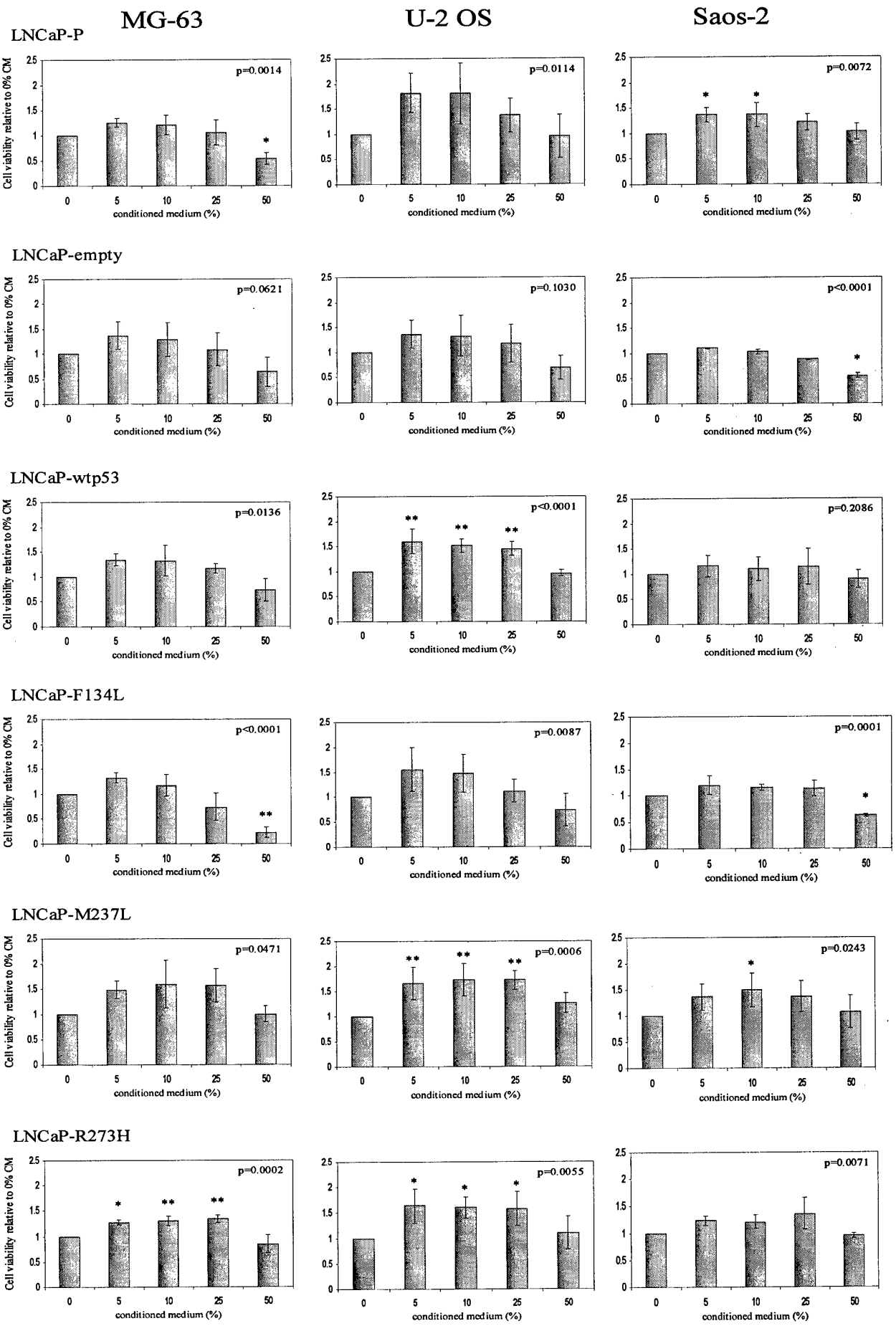
T-medium: DMEM:F-12K, 4:1, supplemented with 5% FBS, 3 g/L sodium bicarbonate, 5 μg/mL insulin, 13.6 pg/mL triiodothyronine, 5 μg/mL transferrin, 0.25 μg/mL biotin, 25 μg/mL adenine.

EBM-2 medium: 94% EBM-2 media (Clonetics; a Cambrex Company, USA), 5% FBS (Clonetics), 0.04% Hydrocortisone, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% Ascorbic acid, 0.1% EGF, 0.1 % Gentamycin and 0.4% bFGF.

APPENDIX 2

Figures 1-11

Figure 1: Proliferation assays. Osteosarcoma cells were treated for 7 days with various concentrations of conditioned medium (CM) from the six LNCaP cell lines. Proliferation was determined using the WST-1 system (see text). Results expressed as a ratio of the control (0% CM).



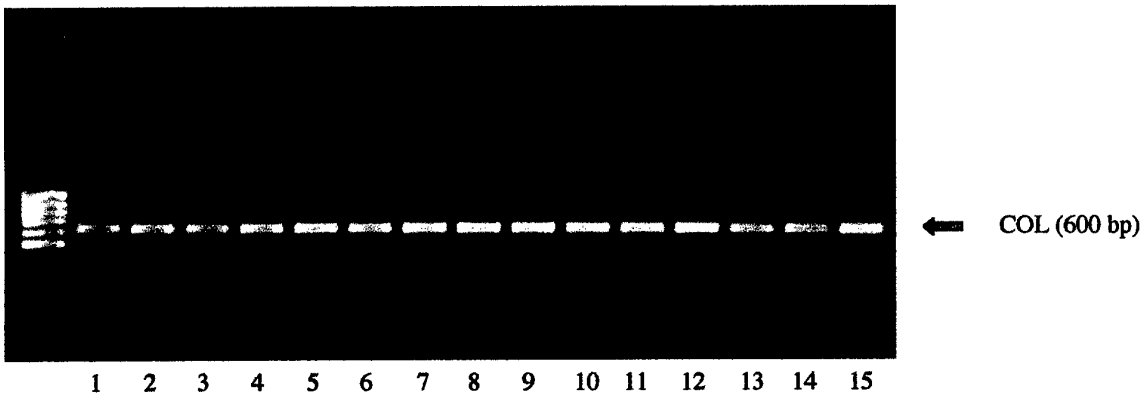
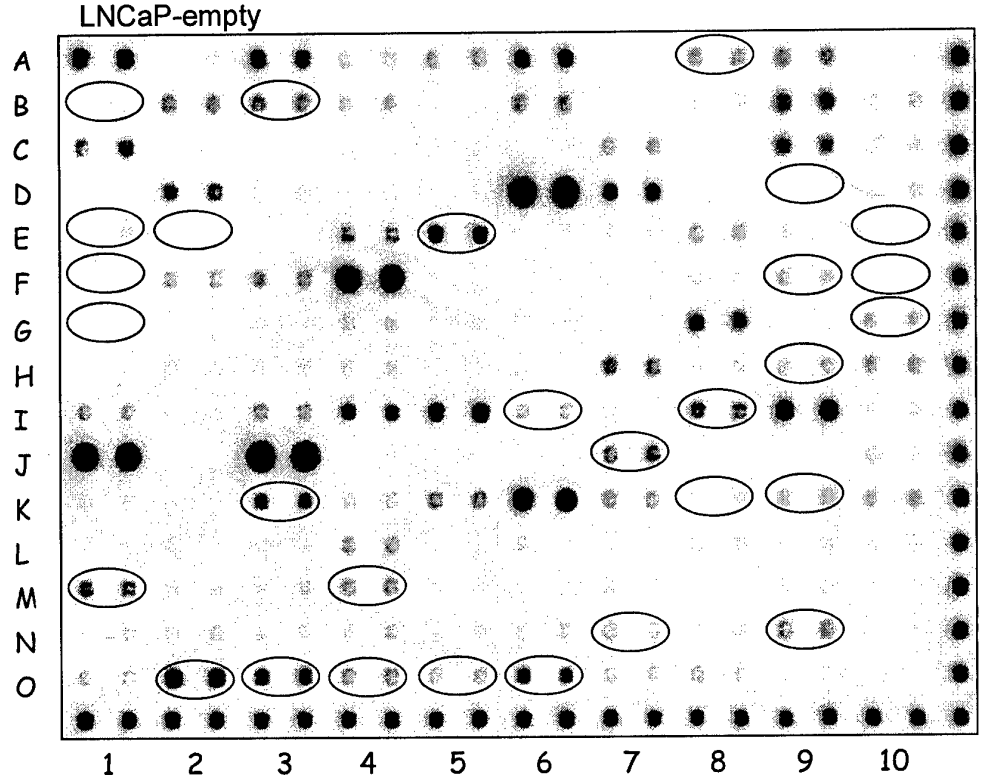


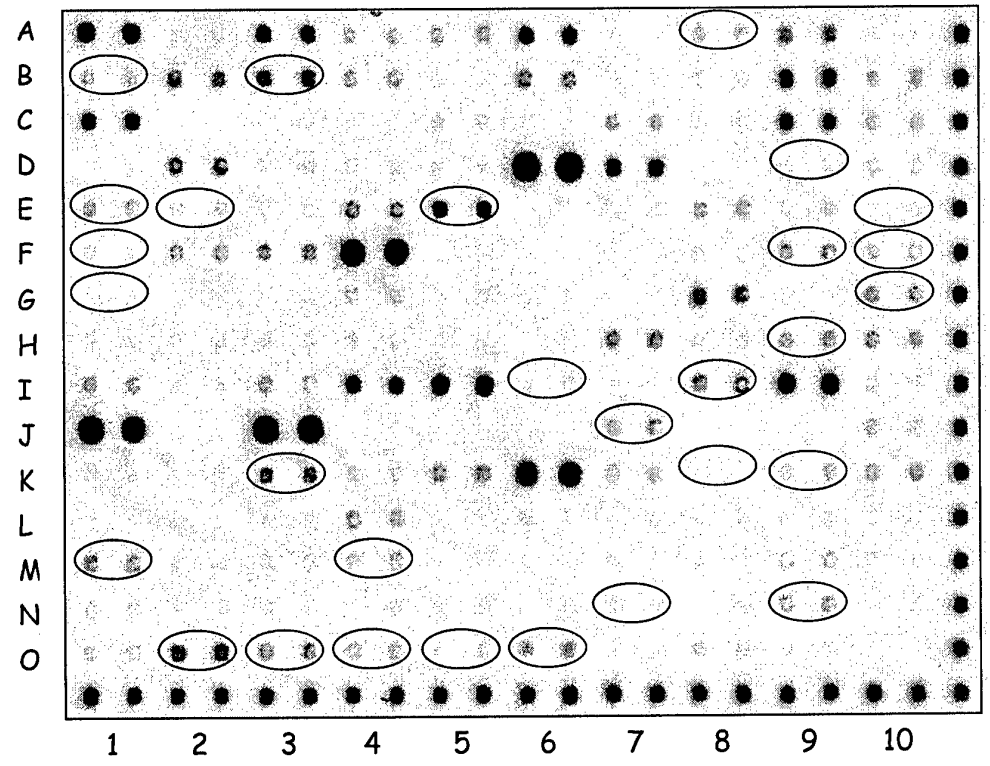
Figure 2: Representative gel illustrating the effects of LNCaP-F134L conditioned medium (CM) upon the levels of type I collagen (COL) gene expression in MG-63 osteosarcoma cells. Cells were exposed to various concentrations of CM for up to 48 h, then harvested for RNA extraction and cDNA production. PCR products were separated on a 1.5% agarose gel. Treatment with 50% F134L for 48 h (Lane 15) increased the levels of COL gene expression by 49.6%, compared with the 0% control (Lane 13), results normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a "house-keeping" gene (data not shown).

Figure 3: Microarray data comparing LNCaP-empty and LNCaP-F134L

LNCaP-empty



LNCaP-F134L



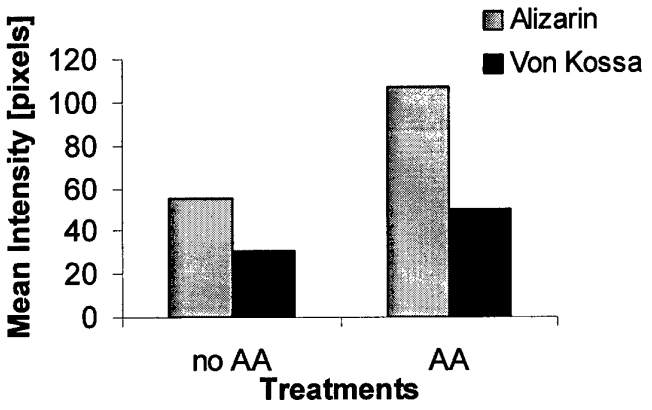


Figure 4: Comparison of Alizarin red S and von Kossa staining methods for analysis of mineralized matrix of the MC3T3-E1 cells and effects of addition of fresh ascorbic acid (AA) to the culture medium.

Figure 5A: MC3T3-E1 cells P9 seeded 7/5/03, cultured in αMEM 5%FBS plus 50 μg/ml ascorbic acid for 4 weeks, then for 2 weeks in 50% T medium 10 mM β-glycerophosphate (top); □MEM 10 mM β-glycerophosphate (bottom)

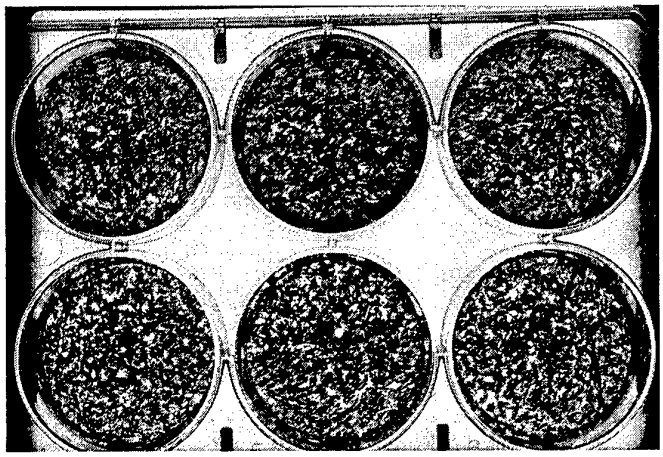


Figure 5B. Alizarin red S staining of mineralized matrix of the MC3T3-E1 cells. MC3T3-E1 cells were cultured in αMEM 5%FBS plus 50 μg/ml ascorbic acid for 4 weeks, then for 2 weeks in either 50% T medium, 10 mM β-glycerophosphate (top) or □MEM 10 mM β-glycerophosphate (bottom).

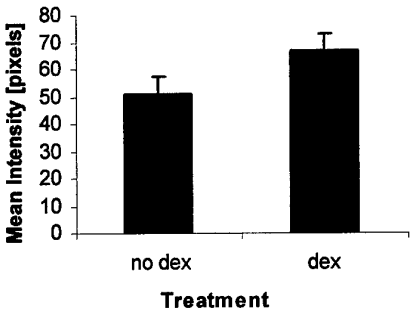


Figure 5C. Effect of dexamethasone treatment during nodule formation stage (i.e. the first 4 weeks of culture) on matrix mineralisation by the MC3T3-E1 cells maintained in 25% T medium during matrix-mineralisation stage (i.e. the last 2 weeks of culture). The cells were cultured in αMEM, 5% FBS, plus additional 50 μg/ml ascorbic acid with 10^{-8} M dexamethasone (dex) or without (no dex) for 4 weeks. Thereafter, cells were cultured in 25% T medium and 10mM β-glycerophosphate without dex for 2 weeks. Mineralized matrix was then stained with Alizarin red S and analysed by densitometry. Results from 3 independent experiments are expressed as mean ± SD.

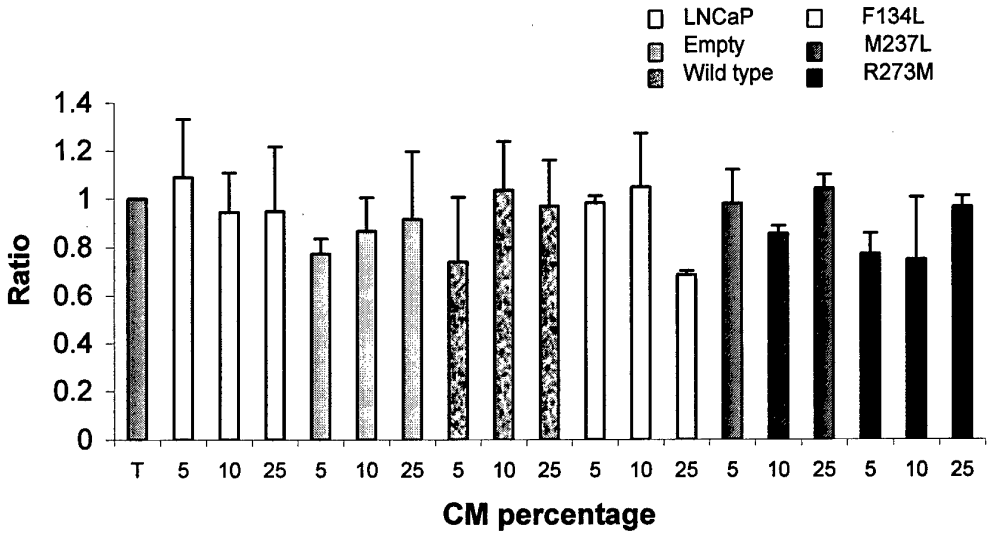


Figure 6: Effects of CM from LNCaP-P and LNCaP transfectants overexpressing wtp53 or mutant p53 on matrix mineralization by MC3T3-E1 mouse calvarial cells. Results of 2 experiments are expressed as mean $\pm$ SD. The work was performed as described in Methods, section *xv*.

Figure 7 : Effects of CM from LNCaP parental and transfected lines on osteoclastogenesis. Methods are as described in part *xvi*.

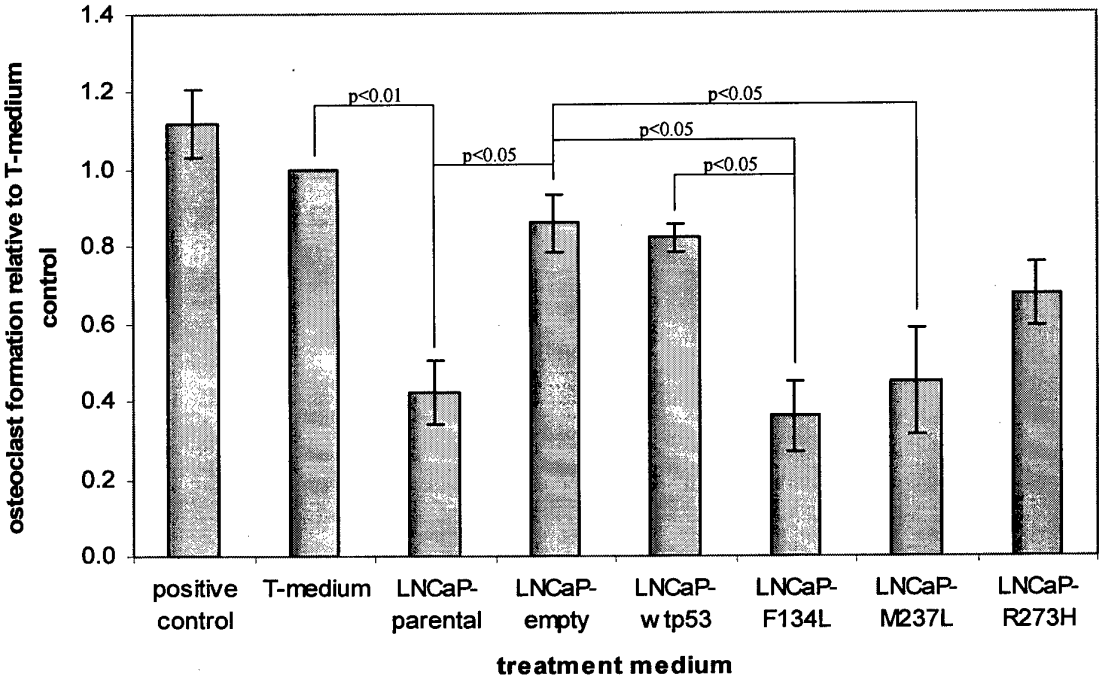


Figure 8: The BMhTERT were seeded at densities from 250 to 8000 cells per well in mixed media (50% EBM-2 and 50% T-media, ■) or full media (100% EBM-2, ▲). The ideal seeding density for the mixed media is 2000 cells per well.

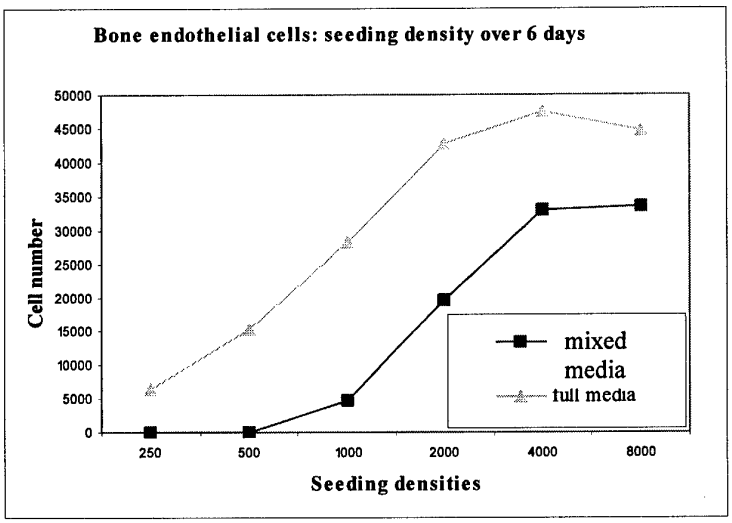


Figure 9. The effects of LNCaP-derived conditioned medium on the proliferation of HUV-EC-C cells. A: LNCaP-parental; B, LNCaP-empty; C, LNCaP-wtp53; D, LNCaP-F134L; E, LNCaP-M237L; F, LNCaP-R273H. Results are represented as ratios when compared with untreated control and are the mean ± s.e.m. of three independent experiments. *, $p<0.05$ when compared with untreated control.

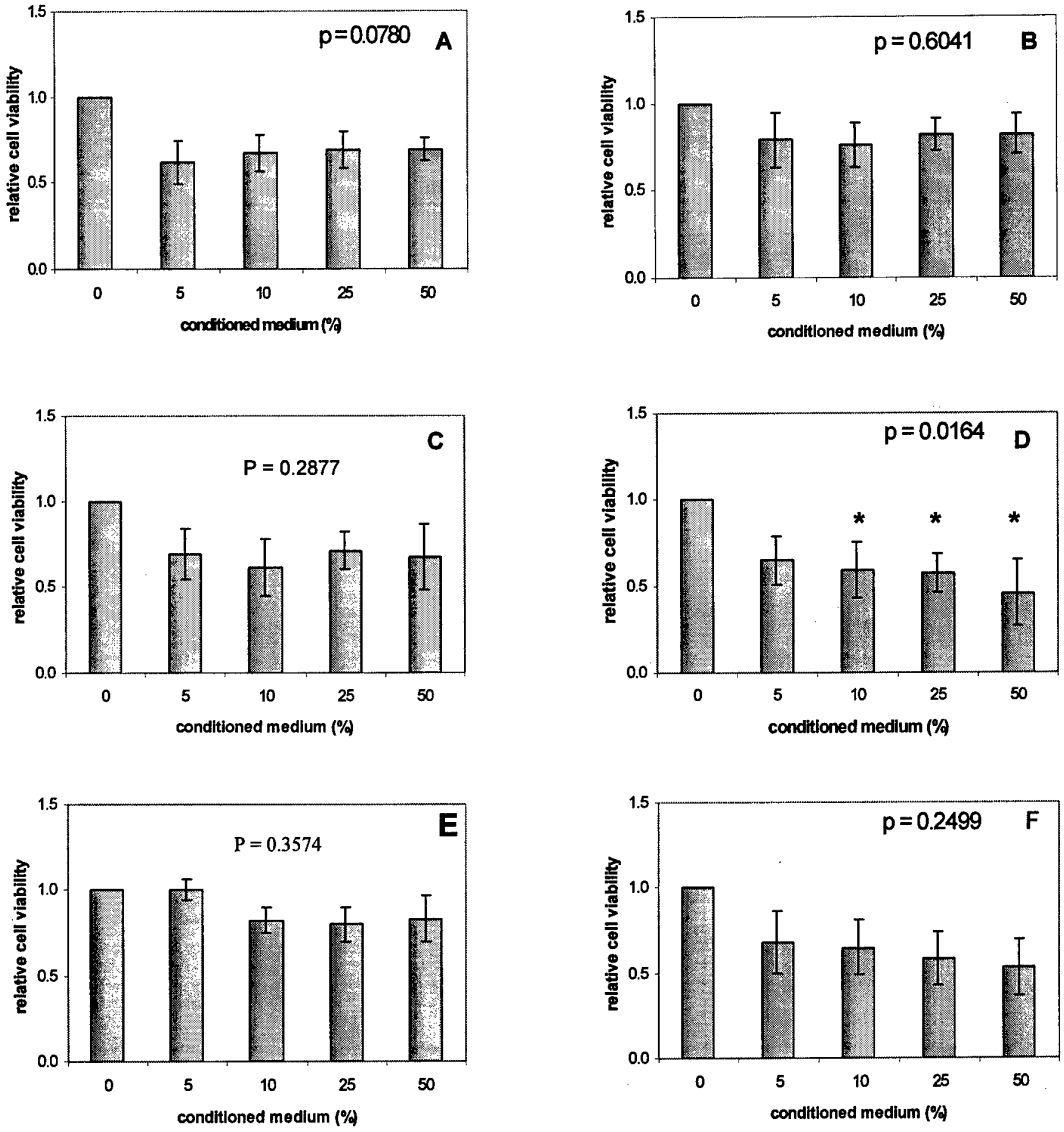
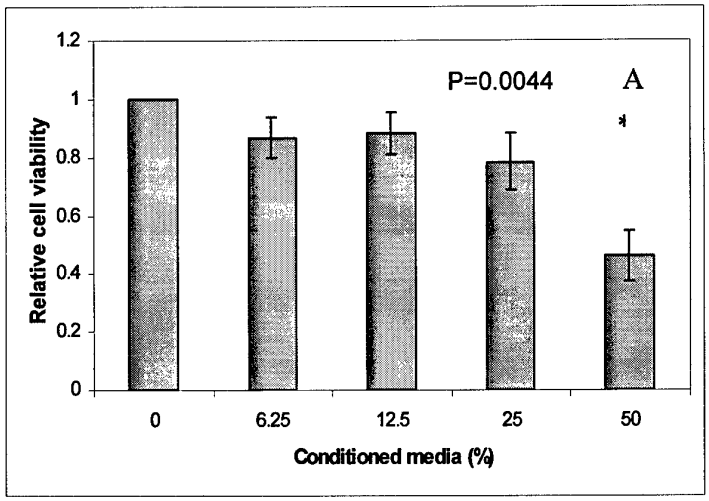
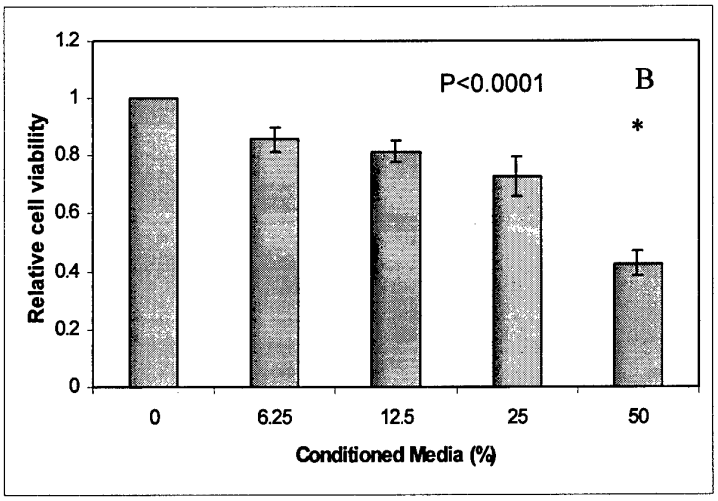


Figure 10. Effects of LNCaP-derived conditioned medium on the proliferation of BMhTERT cells.

A: LNCaP-empty; B: LNCaP-R273H. Results are shown as ratios when compared with untreated control and are the mean ± s.e.m. of three independent experiments. *, $p<0.05$ when compared with untreated control. No significant differences were found when each percentage of the conditioned media was compared between the two cell lines.





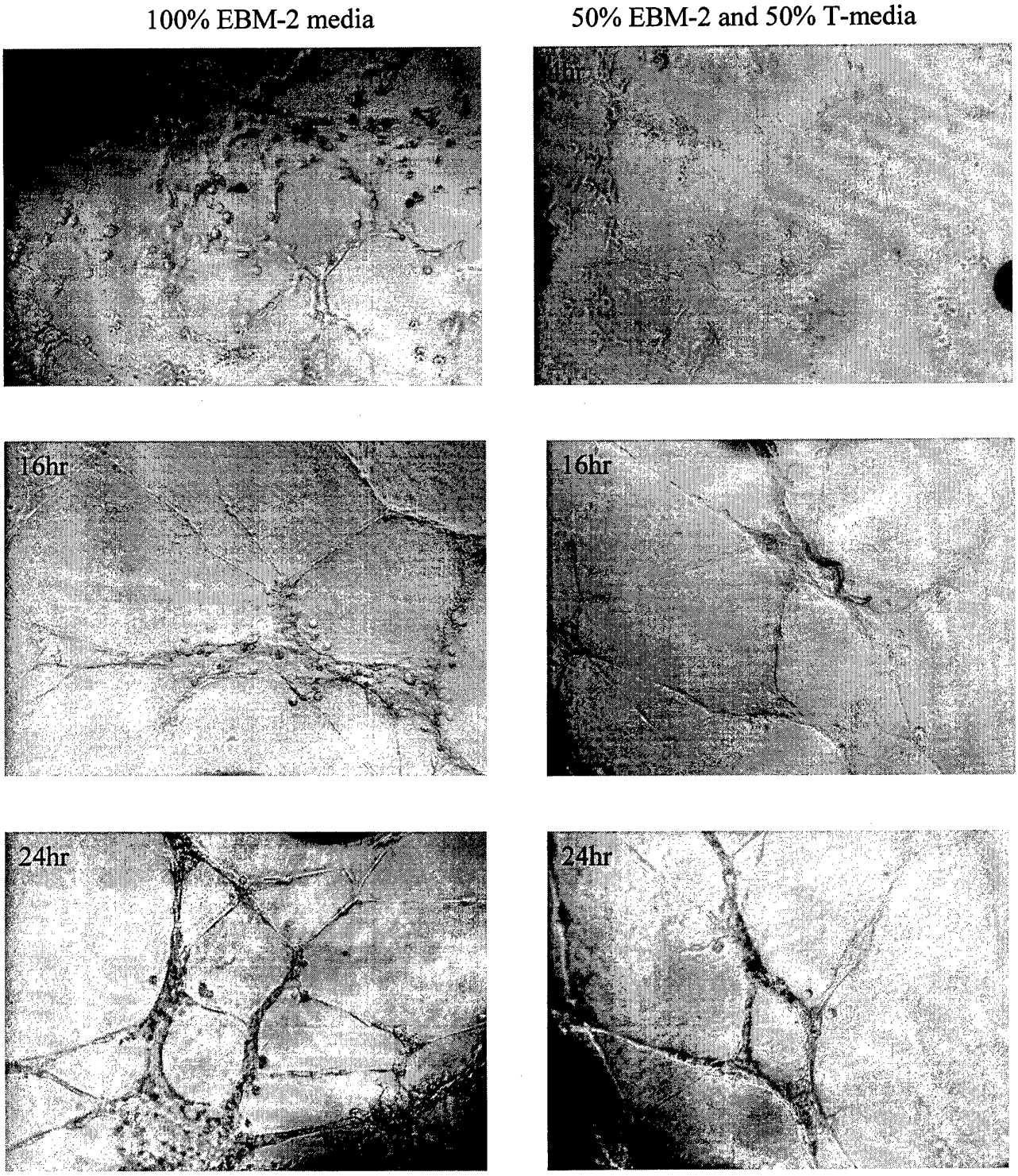


Figure 11: Tube formation by BMhTERT cells plated at 20,000 cells/well in Matrigel. Comparision of 100% EBM-2 medium and mixed medium containing 50% EBM-2 and 50% T-media without FCS effect. The images were taken 4h, 16h and 24h after seeding. Tube formation is apparent by 16 h with thickening by 24h. There is markedly less tube formation in the mixed medium compared to the 100% EBM-2 medium.

APPENDIX 3

Letters concerning SCID mice

Institute of Oncology

University of New South Wales
Prince Henry / Prince of Wales Hospitals
Oncology Research Centre
Director: Professor Pamela J. Russell

10h November, 2003

Dr David Pass,
Animal Resources Centre,
Perth,
WA

Dear David,

Re: SCID Mice

In 2002, we purchased several SCID mice from you to determine the methodology for and take rate after intratibial implantation of prostate cancer cells. Two cell lines were used, LNCaP-parental cells, or LNCaP that had been stably transfected with an empty vector, known as LNCaP-EMPTY; these cells were expected to show similar take rates. We had also prepared stably transfected cell lines from LNCaP that overexpressed wild type p53 (LNCaP-WT) or three different mutations of the p53 gene (F134L, M237L, R273H). After intratibial inoculation of cells, the mice were followed by palpation and by estimations of human prostate specific antigen (PSA) in their sera, taken at 3 weekly intervals. Mice were sacrificed later, generally 18 weeks (39/42) post injection, and their rear legs (control and tumour-injected) were examined by X-ray using a Faxitron. The take rate was established through PSA testing as between 50-70% and by X-ray analysis confirmed by H&E staining as around 50-60%.

Based on these data, more SCID mice were ordered for 2003, and injected with the same lines as above, or with other lines expressing the mutations of the p53 gene described above (Table 1). We would have anticipated that cell lines with mutant p53 would show a higher take rate than those with wt p53 (LNCaP-parent, EMPTY and wt). We also purchased a Faxitron machine to be maintained in the mouse room, so that the mice could be studied periodically by X-ray analysis without having to kill them first. We hoped to obtain growth curves for the various cell lines.

The SCID mice started to lose weight suddenly (see Figure 1), and to become sick, both those injected and those that had been housed separately and had not been injected. In the first mice that were sacrificed, a white obstruction was seen in the gut. However, in later mice, this did not occur. Within each group, shown in chronological order in which the experiments were done, the mice had to be killed because of rapid loss of weight, and the experiments could not be taken out to 18 weeks. It was also observed that as the mice aged, they became increasingly difficult to bleed, and the blood was very dark and viscous. In addition, the mice appeared to be dehydrated, and formed sores easily (e.g., after tail bleeding or after fighting) that did not heal in the normal fashion. The mice that did survive until the end of the experiment were not "healthy" – they appeared to be lethargic and were found on dissection to have pale or blotchy kidneys. A post mortem was done on two mice, one non-experimental

Reply to: **Oncology Research Centre**
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Table 1: Results in mice injected intratibially 2003

Cell line injected	n	Number with PSA >1ng/mL	Time to death (weeks)
LNCaP parent	10, group 3	0/10	9, 11, 12, 14, 15, 15, 15, 17, 17, 18 Median:15
LNCaP EMPTY	10, group 3	0/10	8, 9, 9, 9, 10, 11, 12, 14, 14, 15 Median: 10.5
LNCaP wt	8, group 1	0/8 (2 reached 0.7)	10, 11, 12, 14, 16, 16, 17, 18 Median:15
LNCaP wt	8, group 2	0/10	8, 12, 14, 14, 15, 17, 18, 18, 18, 18 Median: 16
LNCaP F134L	10, group 1	0/10	8, 8, 9, 9, 11, 12, 13, 13, 13, 18 Median: 11.5
LNCaP F134L	10, group 2	0/10	10, 10, 10, 15, 15, 17, 17, 18, 18 Median 16
LNCaP M237L	10, group 1	0/10	9, 10, 12, 12, 13, 18, 18, 18, 18, 18 Median 15.5
LNCaP M237L	10, group 2	1/10	8, 9, 9, 9, 10, 11, 12, 13, 17, 17 Median10.5
LNCaP R237H	12, group 1	0/12	10, 11, 12, 13, 14, 14, 14, 15, 15, 16, 18, 18 Median: 14
LNCaP R237H	10, group 2	0/10	9, 10, 10, 12, 13, 13, 18, 18, 18, 18 Median: 13

and one experimental, by Dr Malcolm France, and in addition, kidneys from 2 mice that had been fresh frozen prior to fixation were also examined. He noted necrosis within the renal papilla (Appendix 1). The kidneys of further mice, taken at sacrifice were examined by renal pathologists, Dr Jonathan Erlich, and Dr Jacob Sevastos, Renal Research Laboratories, Prince of Wales Hospital (see report from Dr Jacob Sevastos) and found to have acute renal tubular necrosis, and the possibility of acute myocarditis. In only one mouse of 100 injected was an elevated PSA seen. Moreover, no changes to the bone were observed when the mice were X-rayed using the Faxitron. Hence the work that we have done has been totally wasted. No tumour takes occurred. It was felt that there was little circulation due to the kidney problems, and that this inhibited tumour take, made it difficult to bleed the mice, and caused their lethargy and difficulties in healing.

We believe that you were aware that there was a problem with the SCID mice, - indeed, you told me that you had had to re-derive the colony twice because of this

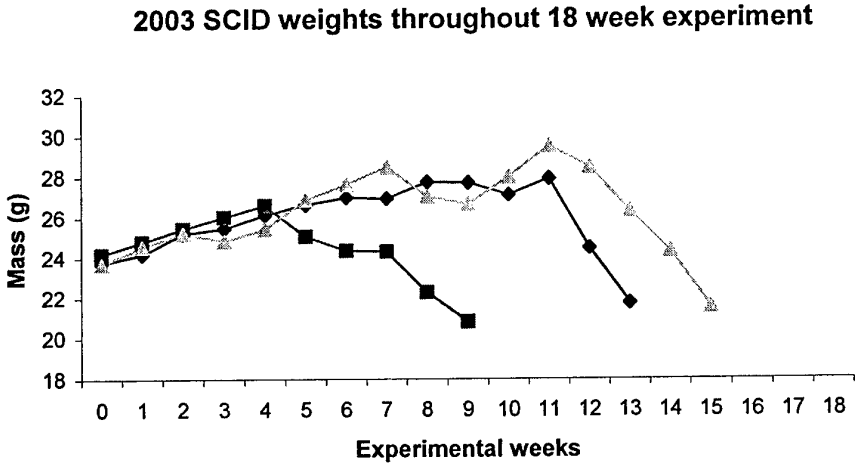
problem, but only after I rang you to see if you knew of any reason why we would be experiencing difficulties with the mice. You explained that kidney infarcts appeared in the new colonies, around 9 months of age, and then began to appear earlier and earlier. We are now faced with knowing how to proceed with our experimental program that is under a strict timeline (funded by Division of Defense, US Army) and are proceeding to test the take rate of control tumours in mice from sources other than ARC.

I have raised this matter with the Board of the BRC, and we feel that it is not appropriate for us to pay for the mice that we have obtained from you in 2003. You should have disclosed that you had had a problem with the SCID mice that could affect our experiments.
We would welcome your comments.
Yours sincerely,

Pamela J Russell

Prof Pamela Russell, PhD

Ms Virginia Fascioli,
Facility Manager,
Biological Resources Centre, UNSW



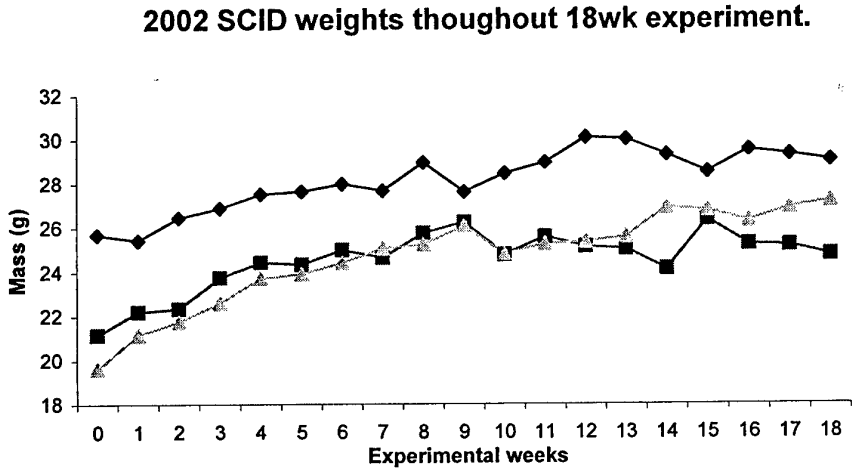


Figure shows the progressive weight loss seen in 3 experimental mice used in 2003 compared with the weekly weights of 3 experimental mice used in 2002.

Dr. Jacob Sevastos
BSc(Med) MB BS(Hons) FRACP
Renal Research Laboratories
Prince of Wales Hospital
Barker St
Randwick NSW 2031

26th November 2003

Dear Sir/Madam,

This report pertains to the evaluation of murine specimens provided by the Oncology Research Group. The specimens originate from SCID mice that have displayed rapid and unexplained mortality, and on gross pathological examination, manifested diffuse renal cortical mottling.

Histological examination of kidney tissue displayed evidence of acute tubular necrosis confirmed by tubular epithelial disruption, cellular swelling, and vacuolation. Examination of cardiac tissue exhibited leucocytic infiltration suggestive of acute myocarditis. Hence, it is feasible that the cardiac pathology predisposed to poor cardiac output and thus decreased renal cortical perfusion, explaining the gross pathological renal appearance at post mortem.

Kind Regards,

Dr. Jacob Sevastos

The University of Sydney

Laboratory Animal Services

Malcolm France BVSc PhD MACVSc
Director

Laboratory Animal Services (B19)
University of Sydney
NSW 2006 Australia
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8 July 2003

Ms Lara Perryman
Oncology Research Centre
Prince of Wales Hospital
Randwick NSW 2031

Dear Lara

As requested, I enclose sections from two scid mice with nephropathy. Note that one (531/03A) also exhibits necrosis within the renal papilla – a finding present in 3 out of the 4 freshly fixed kidneys from scid mice as well as being tentatively identified in the original frozen tissues that you submitted.

You included in the second batch of tissues some specimens from a nude mouse – the kidneys in these were normal. There were, however, some randomly scattered foci of necrosis in the liver (the cause of which was not clear) and a mass of malignant neoplastic tissue (had this been induced experimentally?).

I would be very grateful to receive comments on the scid mouse kidneys from anyone with expertise in renal histopathology.

Yours sincerely

Malcolm France

Malcolm France

Appendix 4.

The quantity of CM stock left from last year's production was not sufficient to cover the experimental needs. New batches of CM were produced to be used in the study of the effects of LNCaP transfectants on mineralisation of collagen by osteoblastic cell lines (task 1D) and on mouse osteoclast differentiation *in vitro* (task 1E).

Method:

CM was produced from the six LNCaP lines: LNCaP, Empty, Wild type, F134L, M237L, R237H. In addition, a batch of CM, to be used in the study of mineralization. Cells were grown to approximately 70% confluency in 150 cm^2 flasks. The medium was removed and the cell layers washed twice with phosphate buffered saline, pH 7.2 (PBS). The cells were then incubated in 25 mL/flask of serum-free T medium for 24 hours. Conditioned medium was prepared from culture supernatants, which were collected, centrifuged to remove any cellular debris, pooled together, aliquotted and stored at -20°C (as prepared in Task 1A, method *i*) Cells from a representative flask were collected and counted. The CM batches produced are shown in Table 1.

Table 1: Conditioned Media (CM) produced from the six LNCaP lines: LNCaP, Empty, Wild type, F134L, M237L, R237H.

Cell line	Passage no.	Date	Volume [ml]	Cells X10^6/flask	Cells/ml CM
LNCaP	49	27/05/03	200	12.12	484800
LNCaP	50	03/06/03	300	10.40	416000
LNCaP	52	11/06/03	400	10.12	404800
LNCaP	53	17/06/03	350	7.36	294400
Empty	13	20/05/03	200	9.89	395600
Empty	14	27/05/03	225	12.21	488400
Empty	16	10/06/03	300	11.50	460000
Empty	17	19/06/03	500	9.60	384000
Wild type	14	22/05/03	200	14.5	580000
Wild type	15	28/05/03	225	10.375	415000
Wild type	17	10/06/03	300	13	520000
Wild type	18	17/06/03	375	10.52	420888
F134L	13	29/05/03	75	11.7	468000
F134L	14	06/06/03	400	11.59	463600
F134L	15	12/06/03	300	12.2	488000
F134L	16	17/06/03	500	11.2	448000
M237L	11	29/05/03	100	10.8	432000
M237L	12	05/06/03	275	11.7	468000
M237L	13	11/06/03	300	13.6	544000
M237L	14	17/06/03	450	11.6	464000
R273M	13	29/05/03	100	12.6	504000
R273M	14	05/06/03	250	15.4	616000
R273M	15	12/06/03	300	13.8	552000
R273M	16	19/06/03	500	13.52	540800
C4-2B	25	24/06/03	525	9.57	382800

Appendix 4 Pools of CM used in mineralisation experiments

	Passage	LNCaP-pool Volume	Date	07/Oct/2003 Cells/ml	Total cells	Cell/ml pooled CM
LNCaP	49	92	27/May/2003	484800	44601600	
LNCaP	49	168	12/Nov/2003	376400	63235200	
LNCaP	53	310	17/Jun/2003	294400	91264000	
LNCaP	50	244	03/Jun/2003	416000	101504000	
LNCaP	52	293	11/Jun/2003	404800	118606400	
LNCaP	51	222	19/Nov/2002	411600	91375200	
		1329			510586400	384188.4123
		Empty-pool		08/Oct/2003		
Empty	13	92	20/May/2003	395600	36395200	
Empty	14	166	27/May/2003	488400	81074400	
Empty	16	245	10/Jun/2003	460000	112700000	
Empty	17	436	19/Jun/2003	384000	167424000	
Empty	15	126	24/Oct/2002	516000	65016000	
Empty	16	166	31/Oct/2002	320000	53120000	
		1231			515729600	418951.7465
		Wild type-pool		08/Oct/2003		
Wild type	15	128	28/May/2003	415000	53120000	
Wild type	17	128	30/Oct/2002	224000	28672000	
Wild type	17	240	10/Jun/2003	520000	124800000	
Wild type	18	324	12/Jun/2003	420800	136339200	
Wild type	14	147	22/May/2003	580000	85260000	
Wild type	16	104	24/Oct/2002	300000	31200000	

		1071			459391200	428936.6947
	F134L-pool			09/Oct/2003		
F134L	14	306	06/Jun/2003	463600	141861600	
F134L	15	224	12/Jun/2003	488000	109312000	
F134L	18	68	07/Nov/2002	656000	44608000	
F134L	17	164	29/Oct/2002	540000	88560000	
F134L	16	429	17/Jun/2003	448000	192192000	
		1191			576533600	484075.2309
	M237L-pool			09/Oct/2003		
M237L	18	94.5	30/Oct/2002	480000	45360000	
M237L	13	19.5	23/Jul/2002	480000	9360000	
M237L	14	379.5	17/Jun/2003	464000	176088000	
M237L	12	187.5	05/Jun/2003	468000	87750000	
M237L	13	228	11/Jun/2003	544000	124032000	
M237L	22	126	03/Dec/2002	480000	60480000	
M237L	17	159	07/Nov/2002	524000	83316000	
		1194			531666000	445281.407
	R273M-pool			10/Oct/2003		
R273M	15	260	12/Jun/2003	552000	143520000	
R273M	16	455	19/Jun/2003	540800	246064000	
R273M	14	58	20/Nov/2002	420000	24360000	
R273M	14	160	05/Jun/2003	616000	98560000	
R273M	16	294	29/Nov/2002	576000	169344000	
		1227			681848000	555703.3415

APPENDIX 5

Poster for The IVth International Conference on Cancer-Induced Bone Diseases, Adam's Mark Hotel Riverwalk, San Antonio, Texas, Dec 7-9, 2003

Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells.

Julie M. Brown[1], Barbara Szymanska[1], Julian M. W. Quinn[2], Elizabeth A. Kingsley[1], Lara A. Perryman[1], Shaun M. O'Mara[1], Paul Jackson[1] and Pamela J. Russell[1].

[1]Oncology Research Centre, Prince of Wales Hospital, Randwick; [2]St. Vincent's Institute of Medical Research, Melbourne, Australia.

Introduction

Mutations in the *p53* gene occur in approximately 94% of prostate cancer (CaP) cases. These mutations are associated with higher Gleason grade, androgen-independence and metastasis. A recent study showed that the same *p53* mutation was present in primary and bone metastatic tumours in CaP patients, suggesting that *p53* mutation in the primary tumour lent a metastatic advantage to the tumour cells.

CaP bone lesions typically exhibit osteoblastic and lytic characteristics, suggesting that CaP cells in bone interact with or direct the activity of osteoblasts and osteoclasts. However, the mechanisms of these interactions are undefined. In this study, we have performed an initial investigation into the ability of CaP cells to modulate the activity of osteoblast-like and osteoclastic cells *in vitro*.

Hypothesis

We hypothesised that CaP-expressed p53 modulates interactions between CaP cells and cells in the bone environment.

Methods

Generation of conditioned medium

Untransfected LNCaP cells (LNCaP-P) and LNCaP cells stably transfected with empty vector (LNCaP-empty) or with vector encoding wild-type p53 (wtp53) or one of three mutant variants (F134L, M237L and R273H, a hotspot mutation found in CaP) were grown to 70% confluency under standard tissue culture conditions and were then passaged using trypsin. The cells were replated at 8 x 10^6 cells/ 150 cm^2 flask and were allowed to adhere for two days. Then, the media were removed and the cell layers were gently washed twice with pre-warmed PBS. Serum-free T-medium was dispensed into each flask and the cells were returned to the incubator for 24 hours. The conditioned medium (CM) for each cell line was pooled and centrifuged to remove cell debris. The CMs were then stored as one-use aliquots at –20 °C until required.

Osteoclastogenesis assay

The femurs of 4-8 week-old C57Bl/6J mice were harvested and the bone marrow was flushed from the femoral cavity using PBS. The cells were plated in quadruplicate into 96-well plates at a density of 3.2 x 10^4 cells/well in αMEM and 10% foetal calf serum (FCS), 100 ng/ml RANK ligand (RANKL), 25 ng/ml CSF-1 and 25% CMs or serum-free T-medium (vehicle control). Media plus supplements were replaced on day 3. Negative control wells contained cells that were treated in the absence of RANKL. Cells treated with αMEM and 10% FCS, 100 ng/ml RANKL and 25 ng/ml CSF-1 were used as positive controls.

Methods (cont'd)

The cells were stained for tartrate-resistant acid phosphatase (TRAP) using a standard histochemical protocol. We defined osteoclasts as cells that were TRAP+ with ≥3 nuclei. Data were expressed as ratios to vehicle control and are represented as the mean ± s.e.m. of 3 independent experiments.

Osteoblast-like cell proliferation

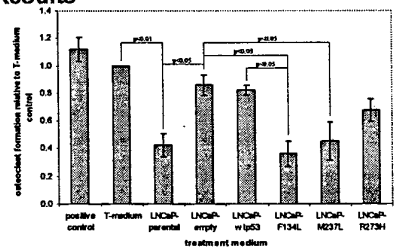
MG-63, U-2 OS and Saos-2 osteosarcoma cell lines, which are thought to model early, mid and late osteoblasts respectively, were cultured under standard tissue culture conditions in supplemented DMEM until 70% confluent. They were passaged and replated into 96-well plates at 300, 200 and 750 cells/well, respectively. They were allowed to adhere overnight, then the media were replaced with treatment media. This consisted of 50% DMEM and 50% CM diluted using serum-free T-medium to produce overall CM doses of 0-50%.

Treatment medium was replaced every 2-3 days for 1 week, then the media were removed and 100 µl phenol red-free medium plus 10 µl WST-1 (Roche) were placed in each well. This assay is a measure of mitochondrial activity and cell viability, and is a measurement of cell number. The plate was returned to the incubator and was read every hour for 4 hours using a plate-reader at 450 nm. Data were expressed as ratios to vehicle-treated control (cells that had been treated with 50% DMEM and 50% serum-free T-medium). Experiments were performed independently 3-5 times.

Statistical analysis

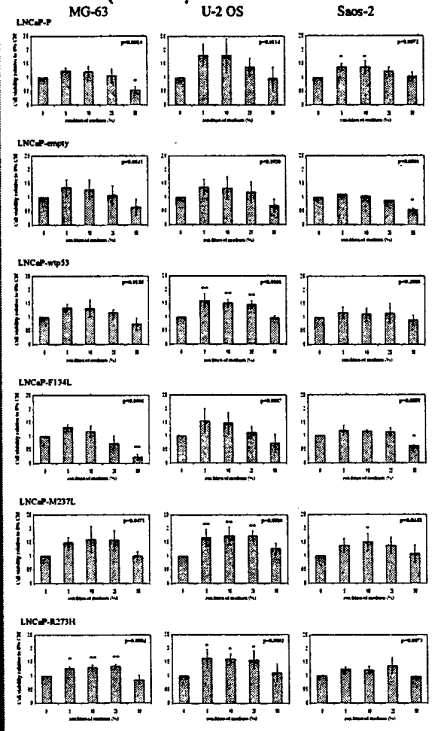
Results were analysed using one-way ANOVA and Tukey's post-tests, where $p<0.05$ was considered significant.

Results



When compared with T-medium, CM from LNCaP-P cells significantly inhibited osteoclastogenesis. When compared with CM from the empty transfectants, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation. When compared with CM from wtp53 transfectants, that from F134L was significantly anti-osteoclastogenic.

Results (cont'd)



CM from LNCaP-P cells inhibited the proliferation of immature osteoblasts and stimulated that of mature osteoblasts. LNCaP-empty CM had little effect on osteoblastic cells. LNCaP-wtp53 CM stimulated U-2 OS cell proliferation only. LNCaP-F134L CM was anti-mitogenic for MG-63 and Saos-2 cells at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS and Saos-2 cell proliferation at intermediate doses. Treatment with LNCaP-R273H CM increased MG-63 and U-2 OS cell numbers at 5-25% when compared with 0% treatment. *, $p<0.05$ and **, $p<0.01$ when compared with 0% CM.

Discussion

The F134L, M237L and R273H mutations are in the DNA binding domain of p53 (amino acids 102-292) and exhibit a dominant-negative phenotype, as determined by promoter trans-activation experiments in CaLu-6 lung carcinoma cells (data not shown). However, in LNCaP prostate cancer cells, where F134L and R273H mutants were dominant-negative, the M237L mutant had a wild-type phenotype and stimulated promoter trans-activation (data not shown). This was mirrored in their *in vivo* growth and metastatic behaviours: F134L and R273H had similar take rates and metastatic growth in lymph nodes as the empty control, whereas M237L and wtp53 take rates and metastases were dramatically reduced (data not shown). These data led us to hypothesise that these LNCaP p53 variants might behave differently in interactions with bone cells.

We found that LNCaP-P cells secrete factors that inhibit osteoclast formation. Empty control cells stimulated osteoclastogenesis over parental cells, suggesting that transfection affected the ability of the cells to modulate osteoclast formation. When compared with empty controls, wtp53 and R273H had no effect on osteoclast formation, whereas the F134L and M237L mutants inhibited osteoclastogenesis, suggesting that these p53 mutations modulate the secretion of factors that influence osteoclast formation.

The changes in osteoclastogenic activity do not occur through increased OPG protein levels, as determined by ELISA (data not shown), and it is unlikely that soluble RANKL plays a role since treatment with CMs in the absence of exogenous RANKL produced no osteoclasts. The most likely explanation, therefore, is that the effects of the CMs on osteoclastogenesis are mediated indirectly through mouse bone marrow stem cells present in the primary culture.

The MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation. The effects on osteoblast-like cell proliferation for all three osteoblast-like cell lines were typically biphasic for most CMs. M237L CM stimulated the proliferation of U-2 OS and Saos-2 cells, whereas R273H was mitogenic for MG-63 and U-2 OS cells, suggesting differentiation stage-dependent effects of these CMs. F134L and empty control CMs had little effect.

Taken together, wtp53 had little effect on osteoclastogenesis but was mitogenic for maturing osteoblasts; F134L inhibited osteoclast formation but had little effect on osteoblasts; M237L inhibited osteoclasts but stimulated maturing and mature osteoblasts; and, R273H had little effect on osteoclasts but stimulated immature and maturing osteoblasts. In summary, these data suggest differing roles for p53 mutations in the response of bone to CaP cells.

Acknowledgements

The authors acknowledge Dr. Sean Downing for the generation and functional testing of the stable LNCaP transfectants. This work was funded by the U.S. Army Medical Research and Materiel Command (grant # DAMD17-02-1-0108).